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Biofabrication

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Contents

1	Biofabrication history and definition — 1
1.1	Aim of biofabrication — 1
1.2	Replacement: engineering prosthetics — 1
1.3	Replacement: transplanting living tissues — 4
1.4	Tissue engineering — 6
1.5	Engineering in medicine — 8
1.6	Biofabrication — 8
1.7	Quiz — 8
	References — 9
2	Scaffold-free biofabrication — 11
2.1	The challenges of biomaterials — 11
2.2	Bioreactors — 12
2.3	From single cells to tissue — 14
2.3.1	Non-adherent mold — 14
2.3.2	Acoustic wave — 15
2.4	Cell sheet — 16
2.5	Biofabrication of spheroids — 18
2.5.1	Hanging drop — 18
2.5.2	Liquid overlay technique — 19
2.5.3	Active rotational method — 20
2.5.4	Microcarrier beads — 21
2.5.5	Magnetic levitation — 22
2.5.6	Acoustic aggregation — 23
2.5.7	Emulsion — 23
2.5.8	Applications of spheroids — 24
2.6	Quiz — 25
	References — 25
3	Organic and inorganic materials for biofabrication — 27
3.1	What is a biomaterial? — 27
3.2	Organic polymers — 28
3.2.1	Poly(styrene) — 28
3.2.2	Poly(ethylene) — 29
3.2.3	Poly(propylene), (PP) — 30
3.2.4	Poly(tetrafluoroethylene) — 32
3.2.5	Poly(amide), Nylon — 33
3.2.6	Poly(carbonate) — 33
3.2.7	Poly(vinyl chloride) — 33
3.2.8	Poly(ethyl vinyl acetate) — 34

3.2.9	Poly(ether ether ketone) —	36
3.2.10	Poly(glycolic acid) —	36
3.2.11	Poly(lactic acid) —	37
3.2.12	Poly(lactic-co-glycolic acid) —	38
3.2.13	Polycaprolactone —	38
3.2.14	Poly(vinyl alcohol) —	40
3.2.15	Polyanhydride —	40
3.2.16	Poly(propylene fumarate) —	41
3.2.17	Polyurethane —	42
3.2.18	Poly(hydroxy alkanoates) —	43
3.2.19	Poly(ether sulfone) —	44
3.3	Synthetic inorganic biomaterials —	45
3.3.1	Calcium phosphate —	45
3.3.2	Bioactive glass —	46
3.3.3	Metal —	47
3.3.4	Inorganic polymers —	49
3.4	Quiz —	52
	References —	52
4	Hydrogels for biofabrication —	55
4.1	What are hydrogels? —	55
4.2	Chemical hydrogels —	56
4.2.1	Poly(acryl amide) —	57
4.2.2	Poly(ethylene glycol) —	58
4.2.3	Gamma irradiation —	59
4.2.4	Protein crosslinking —	60
4.2.5	Photocrosslinking —	61
4.2.6	Click chemistry —	63
4.3	Physical hydrogels —	65
4.3.1	Synthetic physical hydrogels —	65
4.3.2	Natural physical hydrogels —	66
4.3.3	Polysaccharide-based (vegetal) —	67
4.3.4	Polysaccharide from microorganism —	69
4.3.5	Polysaccharide from animal —	71
4.3.6	Protein-based hydrogels —	72
4.4	Quiz —	74
	References —	75
5	Synthetic and natural extracellular matrix —	79
5.1	What is the ECM? —	79
5.2	The composition of the ECM —	80
5.2.1	Glycosaminoglycans —	80

5.2.2	Proteins —	81
5.2.3	Proteoglycans, PGs —	81
5.3	The tissue repartition of ECM components —	84
5.4	Natural ECM for biofabrication —	85
5.5	Synthetic ECM for biofabrication —	86
5.6	Quiz —	88
	References —	88
6	Surface functionalization and topography for biofabrication —	91
6.1	Surfaces for cell culture —	91
6.2	Surface plasma treatment —	92
6.3	Plasma polymerization surface coating —	94
6.4	Photolithography to create shapes on surfaces —	98
6.5	Nanopillar and nanoneedles —	99
6.6	Soft lithography —	101
6.6.1	Replica molding —	101
6.6.2	Capillary molding —	102
6.6.3	Microcontact printing —	103
6.6.4	Microtransfer molding —	103
6.7	Microfluidic devices —	104
6.7.1	Laminar flow —	104
6.7.2	Cell sorting —	105
6.8	Organ-on-a chip —	105
6.8.1	Simulated organs —	108
6.9	Quiz —	109
	References —	109
7	Porous scaffold —	113
7.1	What is porosity? —	113
7.2	Porogen-induced pores —	115
7.2.1	Foam-based porous scaffolds —	115
7.2.2	Crystal-induced porous scaffold —	116
7.2.3	Porogenic solvent —	118
7.2.4	Water as a porogen —	119
7.2.5	Supercritical fluids —	120
7.2.6	Block copolymer phase separation —	121
7.3	Microfiber-based scaffolds —	121
7.3.1	Dry spinning —	122
7.3.2	Melt spinning —	122
7.3.3	Wet spinning —	122
7.3.4	Fiber bonding —	123
7.3.5	Hollow fibers —	124

7.4	Nanofibers fabrication —	125
7.4.1	Electrospinning —	125
7.4.2	Advanced electrospinning —	127
7.4.3	Melt electrowriting —	127
7.5	Cell organization on and in porous scaffolds —	128
7.5.1	Porous scaffold and the immune response —	129
7.5.2	The impact of fiber size —	130
7.6	Quiz —	131
	References —	131
8	Design strategies in biofabrication —	135
8.1	What is design, and what is not design? —	135
8.2	The design processes —	136
8.3	The design approach in biofabrication —	137
8.3.1	Biomimicry —	137
8.3.2	User-centered —	138
8.3.3	Design theory —	139
8.3.4	Morphogenic approach —	140
8.4	The tools for designing objects —	141
8.4.1	Computer-assisted design —	141
8.4.2	Industrial drawing approach —	142
8.4.3	Parametric design approach —	143
8.5	Quiz —	145
	References —	146
9	Bioprinting —	147
9.1	Manufacturing —	147
9.2	3D printing —	148
9.2.1	Stereolithography —	148
9.2.2	Selective laser sintering —	149
9.2.3	Fused deposition modeling —	149
9.2.4	Digital light processing —	150
9.2.5	Multijet fusion —	150
9.2.6	Polyjet —	150
9.3	3D printing for medical applications —	151
9.3.1	Metal printing —	152
9.3.2	Plastic printing —	152
9.4	3D bioprinting —	153
9.4.1	The materials of bioprinting —	154
9.4.2	The bioprinting methods —	155
9.5	<i>In vivo</i> 3D bioprinting —	158
9.6	Pharmaceutical 3D printing —	160

9.7	4D Printing —	161
9.8	Imaging 3D object —	162
9.8.1	Widefield microscopy —	162
9.8.2	Laser microscopy —	162
9.8.3	Light-sheet microscopy —	163
9.8.4	Tomography —	163
9.8.5	Scanning electron microscopy —	164
9.9	Quiz —	165
	References —	165
10	Applications of biofabrication —	169
10.1	The start of an industry —	169
10.1.1	Tissue model —	169
10.1.2	Meat production —	169
10.1.3	Living materials —	170
10.2	Industrial processes —	170
10.3	Sterilization —	171
10.4	Quiz —	172
	References —	172
Index	—	173

1 Biofabrication history and definition

1.1 Aim of biofabrication

During our life, most individuals will go through injuries or diseases that may lead to the malfunction of vital organs. A dream as old as humanity is to repair injured or damaged tissues. From this dream many myths have emerged over our history, depicting heroes and half-gods that were invincible because of their tissue regeneration capacity. Contrary to the axolotl, lizard, or salamander (figure 1.1) which can regrow limbs or only their tail, the human body has a limited regeneration capacity. Although cells are renewed within organs during our life, for instance, the skin cells are replaced every 39 days, bones are remodeled, and a full turnover of the skeletons takes about 10 years. Conversely, some of our tissues do not regrow past a certain age. As an example, tendons, will not regrow or repair past the age of 10. This means that past this time you will have to keep these tissues, through wear and tears and injury. So, the body regeneration capacity is quite limited, and we are not able to regrow a full organ such as a limb or a finger. Fueled by the salamander's capacity to regrow a fully functional limb, our dream of repairing injury is still alive. But, today, thanks to technology and progress in science that has let us better understand our biology, the dream of invincibility has now shifted from a myth to a technological race to fabricate new organs.

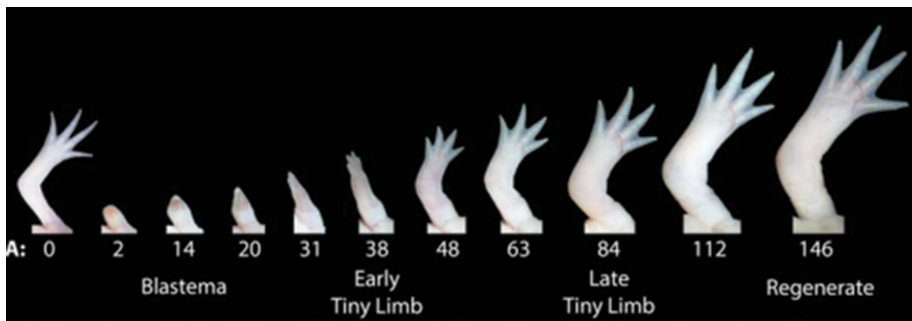


Figure 1.1: The regeneration of the salamander limb after amputation. It takes approximately 4 months for the limb to fully regrow, a dream for humans. Picture from Wells et al. [1].

1.2 Replacement: engineering prosthetics

It is only since the seventeenth century with Robert Hook that we have acquired the notion of cells and only since the 1910s with the work of Burrow that we can reliably culture mammalian cells *in vitro*. So, the first attempt at repairing tissue was strictly an engineered approach, and examples were found in Egypt where artifacts of

prosthetics to replace cut toes were found. But the first industrialization of prosthetics was led by Ambroise Paré in the sixteenth century, who is considered as the father of modern prosthetics. Paré lead a hospital in the heart of Paris, where he seeked to heal amputees and soldiers who were coming back from wars. The first idea was to use materials that could be manufactured cost-effectively to be adapted for many patients. This engineering approach used (Figure 1.1a) materials such as wood, leather, and metal hinges to provide articulated prosthetics. Since the beginning, the materials that we use for the replacement of organs play a critically important role and naturally, with the introduction of more effective materials, prosthetics evolved with the emergence of performance materials. The leather-based prosthetics evolved over centuries to incorporate plastic and composite materials that are lightweight and easily customizable. Further development of materials and the introduction of high-performance composites, such as carbon fiber resins, enable the manufacturing of prosthetics that could overcome the natural performance of natural limbs or at least allow prosthetic patients to perform at the highest level. This was particularly illustrated with the 2012 London Olympics performance of the now infamous Oscar Pistorius, a double amputee wearing a prosthetic limb which allowed him to run the 400 m discipline with non-paraplegic athletes [2] (Figure 1.2b). This demonstrates the advances made over the centuries in understanding mechanobiology, the mechanics and movement of the body, and the development of materials that could mimic, and optimize these movements.

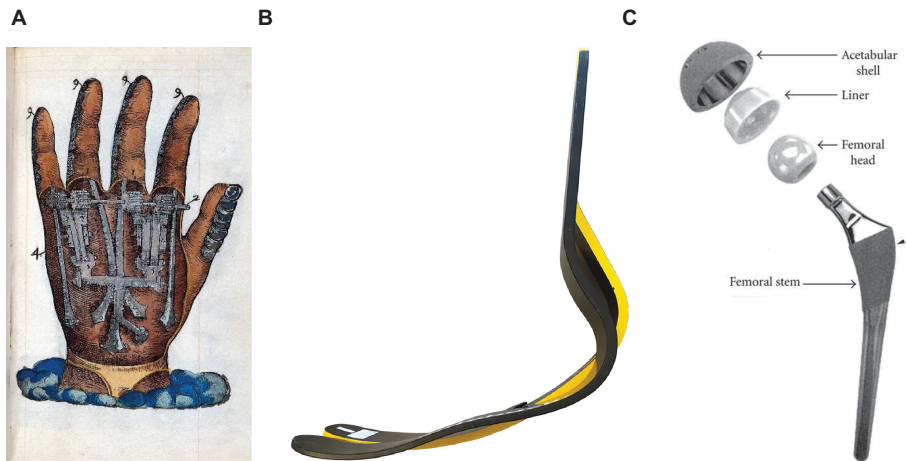


Figure 1.2: Prosthetics through the ages. (A) Illustration of mechanical hand by Ambroise Paré, 1564, (B) Cheetah blade running prosthesis by Össur since 2004 [6]. (C) Total hip replacement components and examples of commercial hip stem implants. Small arrows illustrate porous coating areas using sinter technologies [7].

Along with the development of new materials came the opportunity to develop prosthetic implants, meaning that the prosthesis is not confined outside the body, but

incorporated in the body and thus will interact with the immune system and tissues. For that purpose, biocompatible materials are needed. One of the most common implants is articular implants. In the USA alone, about 7 million people are living with prosthetic implants such as the knee, or hip implants [3] (Figure 1.2c). The implants are made in biocompatible metal alloys, such as titanium alloy or zirconia, and are composed of two parts [4]. One part is hammered in the leg and a second part: a ball is hammered in the hip. These two parts coming in contact are made of the same or different materials to create low-wear low-friction prosthetic through metal-on-metal or metal-on-poly(ethylene) interactions. These implants allow full movement of the legs and can last up to 28 years in place [5].

All these prosthetics approaches led to fully mechanical implants to address the loss of mobility, that is, organs such as legs, toes, or hands that are needed for movement of the body. Going further with implants, it becomes clear that for other organs a simple mechanical implant would not be equipped to do the needed work to restore the function of the damaged organ. The heart, which is a muscle that contracts at different frequency during the day depending on the patient's activity, is quite challenging to replace and need a piece of engineering that is more advanced than for a prosthetic limb. The first implant to address heart defect or cardiac insufficiency is known as a peacemaker. It is composed of a sensor that tracks the cardiac rhythm and an electro-mechanics stimulator to correct arrhythmia (Figure 1.3a). Further engineering approaches in repairing organs have led to the development of artificial dental implants, shoulder prostheses, lumbar disc, or cochlear implants for hearing aids. These prosthetics implants are helping patients to live a better and longer life. But these implants were developed within an engineering paradigm that replace an organ with a machine. These organ replacements are made of metallic parts which in some cases are moving but do not have the same performance as their biological equivalents. Metals can corrode in the aqueous environment of the body; plastic sensors can lose efficacy as there are recognized by the immune system as foreign objects. Like any manufactured product, these implants have an expiration date which depends on the complexity of the implant and its function. For instance, the company Carmat developed a mechanical heart that can fully replace a living heart (Figure 1.3b). However, highly advanced mechanical machinery needs constant supervision and revision, which is not possible with implanted equipment. The current operational time of this mechanical heart is 5 years and is therefore used for patients awaiting heart transplant as a provisory measure. Solutions to overcome the limitation of a mechanical or mechatronic engineering approach would lead to a biological equivalent of the defected organ or injured organ.

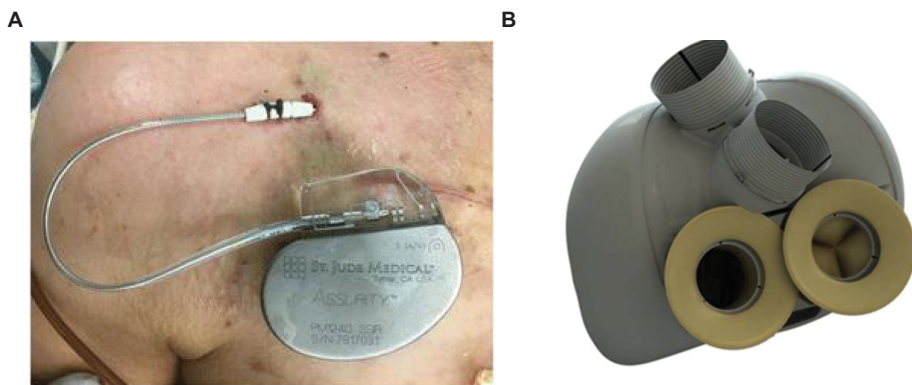


Figure 1.3: Implant for internal organs. (A) Permanent-temporary pacemaker (PTPM): an active-fixation, single-chamber pacemaker lead is fixated to the right ventricle apex or septum and connected to an external pulse generator on the skin [8]. (B) Compliance bag surrounding the prosthesis; atrial suture flanges and ejection conduits [9].

1.3 Replacement: transplanting living tissues

The dream of organ regeneration is as old as our civilization and still alive today. Illustrated by the mythological figures of Prometheus for the Greeks and Tityus for the Romans. Both Prometheus and Tityus's liver was eaten by a crow every day. The liver would regenerate and every night the crow would come again to eat their liver. Although Romans nor Greek new about the regenerative capabilities of the liver, these myths highlight our ancient desire for regeneration. Combining the advances made by surgery and the development of industrial processes further lead to the establishment of the idea of engineering humans, which is best illustrated by Mary Shelley's *Frankenstein* considered as the modern myth of Prometheus. Even today, the myth of regeneration is still alive in our culture and often illustrated in movies and comic books where the protagonist is depicted with the capability to regenerate body parts upon injury. But how far are we from realizing this dream?

Early attempts of using biological tissue to regenerate injured tissues have been found in old manuscripts originating from India in the *Sushruta Samhita* which are dated from 1000 BC [10]. In this document, a surgical procedure to transplant skin flaps from the patient to repair the earlobe is described. Influenced by the Indian literature, Tagliacozzi published the arm-to-nose skin transplantation believed to have been developed by the Branca and Vianeo family (Figure 1.4). This procedure called the Italian method was developed in the fifteenth century [11]. This was commonly used in Italy for the reconstruction of cut noses from fencing accidents. This is considered as the first tissue transplantation of modern medicine bringing the idea of taking tissue from a part of the body that is non-damaged to replace the injured tissue.



Figure 1.4: Illustration from the sixteenth-century technique of rhinoplasty showing the transplantation of skin from the arm to the nose. From *De Curtorum chirurgia per insitionem* (1597) by Gasparo Tagliacozzi.

Further iteration led to taking tissue from cadavers which was first achieved by Girdnerin in the nineteenth century [12]. These were the first transplantations of tissue which eventually led to the first organ transplantation of the thyroid performed by Kocher in 1881 [13]. Decades later, with the understanding of the immune systems and findings of anti-rejection drugs, other organs followed such as kidney, heart, liver, and lungs. Today, organs transplantation is a logistic challenge that requires coordination between multiple medical centres to promptly match donors with a patient. But the establishment of a transplantation network across countries and territories, while increasing the matching success, has not been able to resolve the organ shortage. Although policies established in Spain for instance, that considers all citizen as donor except if they actively opt-out have allowed decreasing the length of the waiting list, there is still a shortage of organs that will not be resolved soon. Indeed, transplanted organs have a limited lifetime in the patient. Often, organ recipients need to get several transplantations during their life. So, to address the organ shortage, the idea of growing fully functional living transplants has emerged in the late 1990s with the first demonstration of the growing of living cartilage tissue on the back of a mouse was made by

Vacanti (Figure 1.5) The picture made it around the globe, and this started the field of tissue engineering.

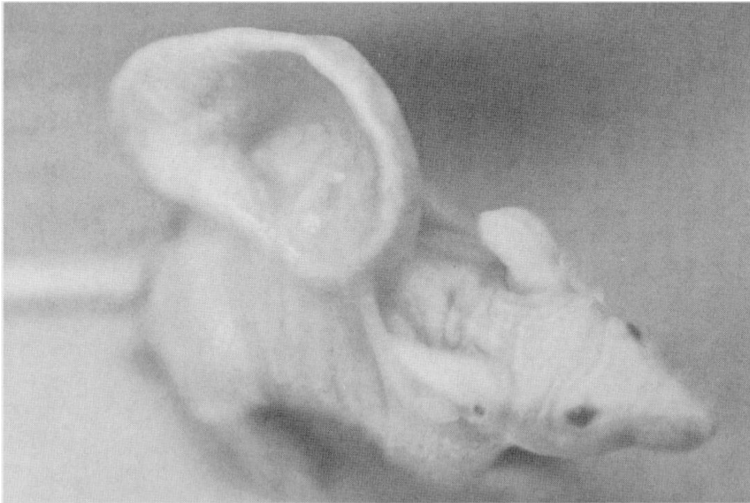


Figure 1.5: The Vacanti mouse is a laboratory mouse that has cartilage tissue grown subcutaneously in what looked like a human ear on its back [14].

1.4 Tissue engineering

Tissue engineering is combining materials science principles with cells biology principles to create living implants. This new approach lets us envision endless possibilities in creating living implants that could replace injured or diseased tissues. Tissue engineering when proposed suggested the possibility to tackle the shortage of organ transplants. If we could make on-demand living implants that are as good as living organs, we would not be limited to cadaver donors to reduce the number of patients on the organ transplantation waiting list. Because tissue engineering proposed to use single cells to grow an organ on a biocompatible material, it brings the possibility to use the patient's cells to create a new organ and create an allograft Table 1.1. Indeed, organ transplantation, because it is using living materials from a foreign system is recognized by the patient immune system as a threat. So, to avoid organ rejection, one needs to match the organ donor with the receiving patient to assure the best compatibility. Additionally, immunosuppressive drugs are needed to trick the patient's immune system into accepting the implant. Using cells that originate from the patient to engineer a replacing tissue opens the possibility to get rid of immunosuppressive drugs after the implantation, thus considerably improving the life of the patient and longevity of the organ. As tissue engineering developed, several successes have demonstrated the

relevance of the field: tissue-engineered bladder [15], or nose-to-knee cartilage transplantation [16]. But these successes have been long to develop and remain for the most part an artisanal task that is difficult to bring to scale. If we want to have mass production, industrialization of the tissue engineering processes is needed.

1.A Types of transplantation

It is key to understand the difference between the different types of transplantation. This table has summarized the type of transplantation possible (Table 1.1).

Table 1.1: Types of transplantation.

Recipient of the transplantation techniques	Donor			
	Animal	Human	Identical twin	Recipient
	Xenograft	Allograft	Isograft	Autograft

1.B Organ systems

In this book, we will talk about the different types of biofabrication techniques to create living implants. It is key to understand the type of biofabrication and what can be created with each type of biofabrication technique, for instance, cells, tissue, organ, and organ systems (Figure 1.6).

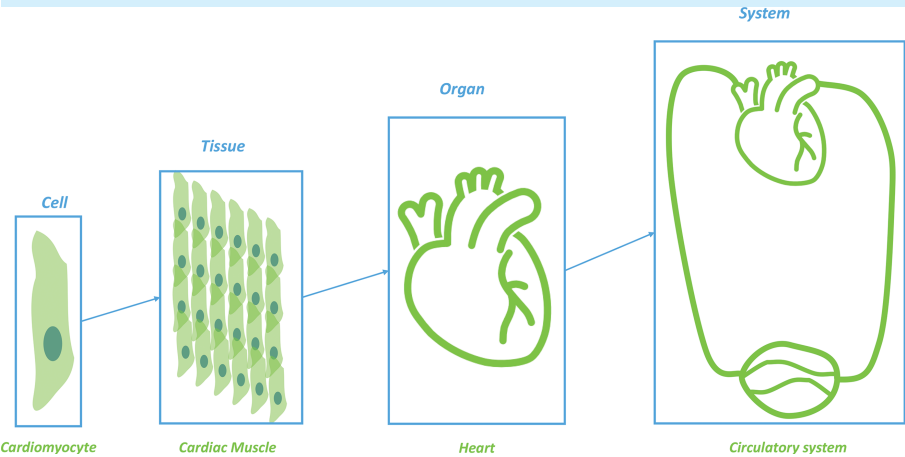


Figure 1.6: The hierarchy of system and cells.

1.5 Engineering in medicine

More and more machines and technical devices have made their way into the operation theaters. Highly engineered and complex tools are now part of surgical procedure: kits containing all the surgical tools to perform a procedure, catheters that are inflatable, analytical instruments such as magnetic resonance imaging or X-ray and bypass machine that can replace kidney, heart, and lung to allow for the surgeon to operate on the organs. The constant development of medical tools has led to more and more advanced instruments. For instance, human-guided robots like the Davinci robots from Intuitive Surgical facilitate minimally invasive surgical approaches, with the potential to perform remote surgery and in the future may be fully automated procedures. Modern medicine heavily relies on the technological advances made in the past decades and is interconnected with engineering.

1.6 Biofabrication

We can see that we have a convergence of engineering, biology, and materials science toward the development of tools and procedures to improve medical care at a mass level. Biofabrication comes at the interface of all these disciplines to propose an industrial approach for the engineering of living implants and biological products. Biofabrication can be defined as the production of complex living and non-living biological products from raw materials such as cells, molecules, extracellular matrices, and biomaterials by using industrial fabrication techniques. That is creating a framework of processes and techniques that will allow for the mass production and standardization of these biological products. The goal of biofabrication is to overcome the limitation of current tissue engineering procedures that have been successful for a single patient but to translate these procedures for the masses and as a therapy that can serve several patients.

In this book, we will present different aspects of biofabrication. The chapters of this book have been built to present methods and concepts of increased complexity in the manufacturing process. Starting with living tissue that is only composed of cells, we will then present methods using scaffolds to support the cell growth. Finally, we will discuss the limitation and challenges associated with the manufacturing of biological products and their translation to the clinical space.

1.7 Quiz



1. When dates the first record of an attempt at medical repair?
2. What is biofabrication?

3. What is tissue engineering?
4. What is the main limitation of organ transplantation?
5. What is the main limitation of prosthetics?
6. In the myth of Prometheus and Tityus, which organ was eaten by crows every night?
7. Which of its organ can the axolotl regenerate?
8. What is an allograft?
9. What is the difference between organ and tissue?
10. What are the requirements of implants?

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2 Scaffold-free biofabrication

2.1 The challenges of biomaterials

Biofabrication approaches often use a biomaterial that is processed into a scaffold that supports the growth and organization of cells into functional tissues. However, this approach is not perfect and has some challenges. So, the hypothesis that it is required to have a scaffold for the cells to grow, is not always supported by experiments. Indeed, cells can generate their scaffold by secreting macromolecules which form the extracellular matrix (ECM) in the tissue. If we can induce the formation of natural ECM by the cells *in vitro*, then we do not need biomaterials to support the cells. Biomaterials, while providing support for the cell to grow, generate new challenges. Some biomaterials can induce an immune response and so the scaffold made up of the biomaterials can be seen as a foreign object and can induce an adverse immune response. The second challenge is that the biomaterials' biodegradability needs to be taken into consideration. For some applications, a degradable scaffold might be beneficial, for others not. If a biodegradable scaffold is required, then the degradation rate needs to be optimized for each application. Finally, the selected biomaterials need to be sterilizable by conventional industrial sterilization methods such as autoclave, gamma irradiation, or ethylene oxide. Beyond these challenges, scaffold enables to provide support and a shape to direct the cell growth. This support can be crucial to provide mechanical support for the cells to grow and mature. In the absence of mechanical support, a higher amount of cell is required to create mechanical support based on the secretion of natural ECM macromolecules such as hyaluronic acid, proteoglycan, and collagen.

2.A Extracellular matrix (ECM)

The ECM is a three-dimensional network that is composed of macromolecules, minerals, growth factors, and enzymes (Figure 2.1). The network is created by fibrillar and hydrogel-forming macromolecules such as collagen, other hydrogel-forming macromolecules such as fibronectin, or non-hydrogel forming such as heparin and hyaluronic acid. Also present in the hydrogel is cell-generated enzymes such as matrix metalloproteinase that are metal activated (usually zincs) that can cleave the macromolecules of the ECM. Other cells-generated signaling molecules are present in the ECM such as growth factors that can interact with the macromolecules like heparin. The ECM is created by the cells and it is critical for binding cells together and creating functional and mechanically stable synthetic tissues.



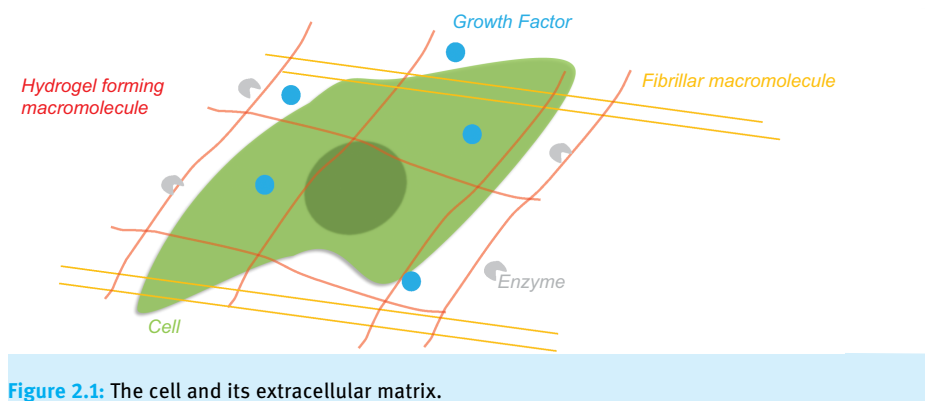


Figure 2.1: The cell and its extracellular matrix.

2.2 Bioreactors

At the laboratory scale, cells are cultivated in sterile containers that provide adhesion for cells. These containers, coming in different shapes and volumes, are used for low volumes, cells are generally cultivated in microplates that are constituted of wells capable of holding volumes as low as a couple of microliter up to several milliliters. These plates are made of poly(styrene) that is gamma irradiated to induce cell adhesion and sterilization in a single process. The microplates are designated by the number of wells typically from 96 to 6 wells per plate at the laboratory scales translating in volumes ranging from 100 μL to 10 mL. Then for higher volumes, T-flasks are used. These consist of a trapezoidal shape that can hold volumes from 40 to 400 mL. The cells are cultivated on the bottom of these flasks which can be stacked in the incubator. These containers are mainly used for adherent cells such as fibroblast (skin cells) or endothelial cells (blood vessel cells). For cell conditions where adhesion is not required, one can use non-adherent conical tubes of different volumes. These tubes are made of poly(propylene) and come in two different shapes of tube: Eppendorf's tubes ranging from 0.5 to 2 mL and Falcon's tube from 15 to 50 mL. The conical shape allows for the recovery of cells after centrifugation. These flasks can sustain a centrifugal force of up to 30,000 g and are also used for the recovery of protein and other molecules (Figure 2.3).

In increasing the number of cells, bigger containers are needed, so-called bioreactors. These are also used in a laboratory setup. They are made of poly(propylene) or glass and can range from 125 mL to several liters. Processes optimized with these bioreactors then be scaled up to industrial setup using tanks of several hundred liters. The bioreactor is a cylinders with several opening on the top that allows the changing of the culture media and are equipped with a mechanical stirrer that keeps the cells in suspension. Indeed, without agitation, cells will fall at the bottom of the reactor by gravity. However, some type of cells only grows when they are attached to a support, and it is

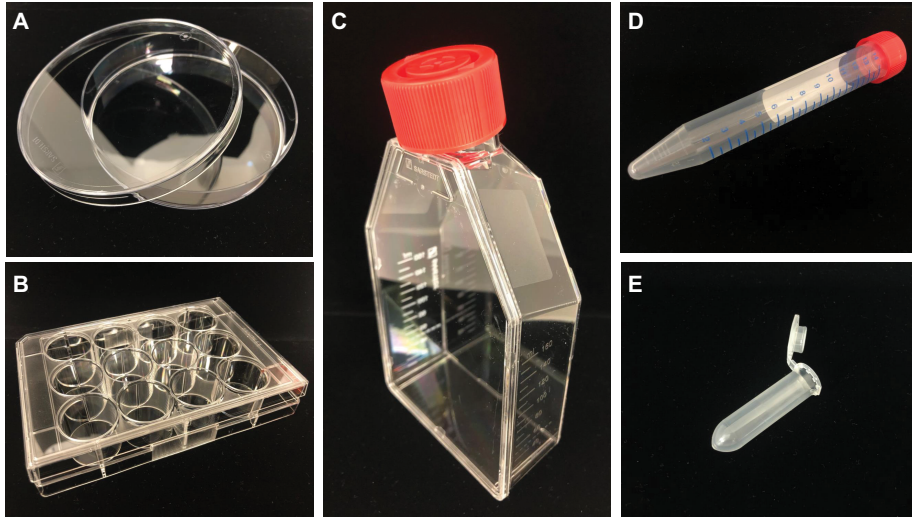


Figure 2.2: Typical plastic ware used in cell biology for the cell culture. (A) Petri dish laboratory and (B) well plate, (C) T flask, (E) Eppendorf's tube and (D) Falcon tubes.

not possible to grow them in suspension. So, to increase the culture surface area from the T-flask planar format but reduce the footprint of the reactors, small particles on which cells can attach are added in the bioreactors. The spheres or particles are usually made of polymers of 500 μm diameter to which the cells can attach, thus increasing the surface area. Commercial setup to further increase the available surface area for cell culture consists of fibrous bioreactors. These are presented as cylinders packed with micrometer diameter and parallelly aligned fibers to which the cells can attach (Figure 2.4). Between the fibers, the cell culture media can be circulated, and the product of the cells culture can be extracted, such as antibodies. The fibers can be plain or hollow, thus further increasing the available culture surface. Inherited from biotechnology and vaccine production, these two techniques allow for scaling up the production of cells and obtaining a large number of cells which can be then used for biofabrication.

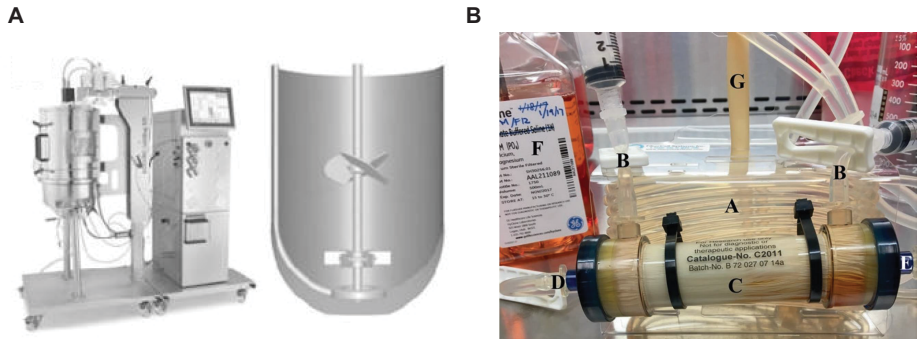


Figure 2.3: Scaling up cell culture. (A) The stirred single-use cell culture bioreactors from BIOSTAT CultiBag STR with a working volume of 50 L [1]. (B) Components of a typical hollow fiber bioreactor setup. The entire setup, including the perfusion pump, is within a temperature-controlled cell culture incubator. (a) gas exchange circulator; (b) Luer-Lock harvest and injection ports (shown with syringes attached); (c) hollow fiber cartridge; (d) circulating media inlet; (e) circulating media outlet; (f) complete media reservoir; (g) tubing interface to perfusion pump (pump not shown) [2].



2.B Cell surface adhesion mechanism

On tissue culture surfaces such as plastic Petri dishes, the plastic of the surface is treated to be hydrophilic. Molecules present in the cell culture media such as albumin will form a coating on the substrate. Then cells suspended in this media will sediment on top of these coated surfaces and adhere to the adsorbed molecules.

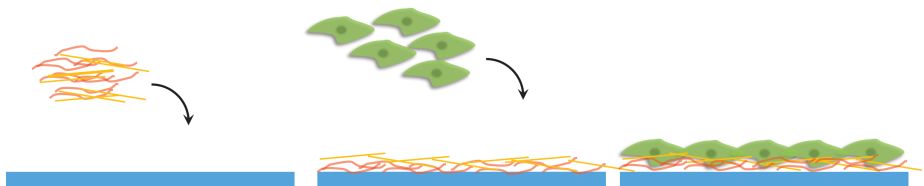


Figure 2.4: Cell-adhesion mechanism. First, protein adsorbs on the cell culture substrate. Subsequently, cells attached to the proteins coating the surface of the cell culture substrate.

2.3 From single cells to tissue

2.3.1 Non-adherent mold

Once a critical number of cells is obtained, we need to organize them into tissue without using a scaffold. Some types of cells can organize by themselves through gravitation into a spheroid when they are cultivated on a non-adherent plate. This is the case for chondrocytes which are cartilage cells and many cancer cells. Others need some help and need to be forced into aggregation to form a tissue. To achieve this, one can use

molds that are non-adhesive which will force cells to find each other and organize into a predictable shape. To do that the mold will restrict space and guide the gravitation of the cells (Figure 2.5). Typically, these molds can be made of poly(dimethylsiloxane) or hydrogel such as agarose. These molds can be obtained by making a negative shape using typical manufacturing techniques or 3D printed molds. The negative mold is then used to make the mold that will get in contact with the cell. This technique is called replica molding and is widely used across different industries to make a molded part. This approach can be used to create cell aggregates in a shape of a ring by using a doughnut mold. More advanced architecture can be obtained such as honeycomb by using a mold with an adequate shape. However, the cells need to be cultured long enough to have produced enough ECM to provide them mechanical strength to sustain their shape once they are removed from the mold. If removed too early, then the cells do not have the time to create the needed ECM macromolecules to maintain the shape and the cell aggregate will collapse.

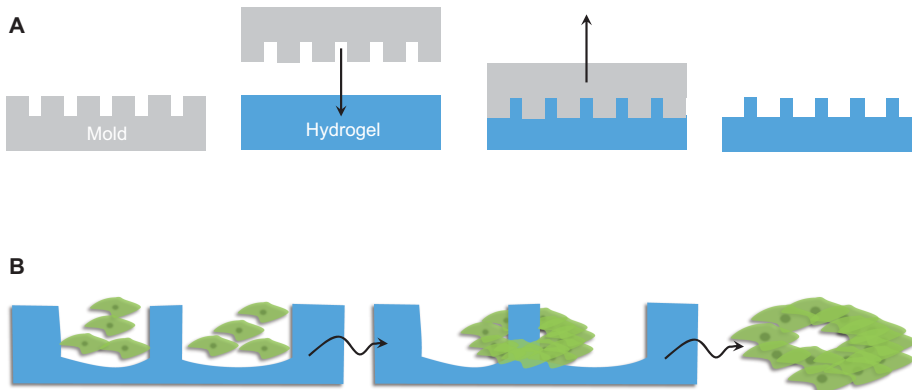


Figure 2.5: Controlling cell organization with a mold. (A) The mold is created using a replica molding technique. A negative mold is manufactured and used to stamp a hydrogel precursor liquid. The hydrogel is set, and the mold is removed leaving its imprint in the hydrogel. (B) Using a circular non-adhesive mold cells (green) can be organized in the shape of doughnuts recreating hollow cellular organization observed in blood vessels for instance.

2.3.2 Acoustic wave

Going a step forward is to get rid of the mold and directly position individual cells precisely without contact. This can be achieved by using acoustic waves to move particles and cells into predictable shapes. This approach is quite interesting, at first, it is highly versatile so the manufacturing can be made instantaneously without the need to pre-fabricate a mold. Second, it reduces the risk of contamination through contact with the mold or contamination with the contact of the mold materials (Figure 2.6). Emerging

from this technology, acoustic wave generators are capable of organizing cells into 3D morphogenic patterns. Such instruments are capable of creating a standing wave that is generated when a wave traveling in one direction is interacting with a wave traveling in the opposite direction. The overlapping wave is called a standing wave which oscillates in time but not in space. By changing the frequency and amplitude of the acoustic waves, the object trapped in the nodes and antinodes can be displaced within the media [3].

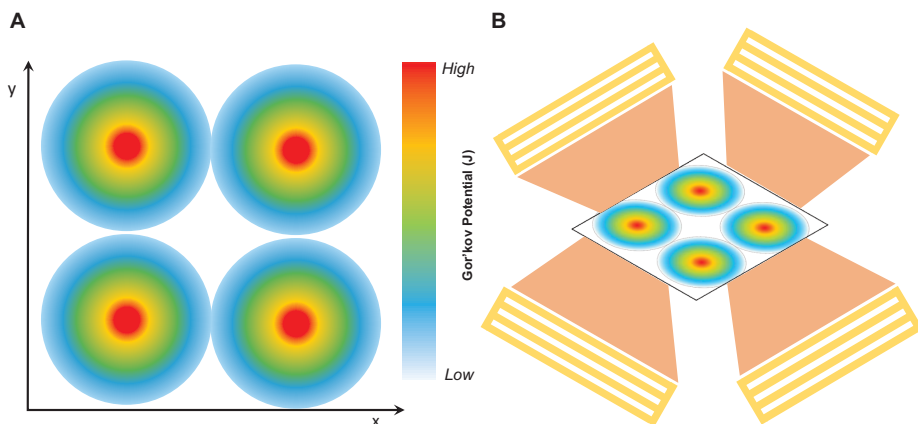


Figure 2.6: (A) The patterns have high and low potentials that permit to precisely position the cells within the nodes of the standing wave. (B) Acoustic wave setup composed of piezo-activated actuator that applies a wave at a set frequency and amplitude. In the container, an acoustic standing wave will be generated by using two opposite piezo generators. The four actuators enable the creation of complex 3D patterns.

2.4 Cell sheet

While 3D architecture is interesting for several types of tissue biofabrication approaches, some therapeutic applications preferably deliver cells as a sheet. As an example, skins replicates can be created and applied as a sheet of cells to cover a wound, but also cardiomyocytes sheets applied as remediation of myocardial infarct. To make cell sheets, one needs to be able to grow cells on a surface, easily achieved using conventional cell culture technique, but to remove the cells as a single mechanically stable living tissue is challenging. One method proposed to do this is by using a temperature-responsive surface coating that lifts the whole-cell sheet together without altering the biology of the cell by using cleaving enzymes such as trypsin.

The material poly(*N*-isopropyl acrylamide) (poly(NIPAAM)) exhibits a lower critical solution temperature (LCST). This means that above the LCST the polymer is hydrophobic. It will then form a thin coating on a petri dish on to which proteins can

attach. Below the LCST, the polymer chains are extended and soluble in water. Which means that the poly(NIPAM) can detach from the tissue culture plate and lift with them the proteins that had attached to it (see knowledge box cell adhesion mechanism). As a result, the cell sheet is lifted with the deposited ECM and can be harvested as a single piece of living tissue (Figure 2.7). Once the cell sheet is obtained, the sheets can be combined to reproduce different cell layers, for instance, the different layers of the skin reproducing the epidermis and the dermis. Using the sheet manufacturing approach applied to cardiomyocyte allows for creating several layers of cardiac cells that have enough mechanical strength to be sutured to the heart.

Going further with the sheet technique of cardiac tissue, one can try to reproduce the striation anatomy of the cardiac muscle. To reproduce the linear pattern of the cardiac tissue, cardiomyocytes can be cultured on a mold that forces the cells to align into natural striation. On poly(dimethylsiloxane), PDMS, molds, the cells cannot adhere and can be then lifted and detached into a mechanically stable sheet of cells that can be sutured into a cardiac patch. In this patch, the cells can be stimulated to beat like a cardiac muscle.

Another application for cell sheets is skin sheets. The technique consists in creating layers of fibroblast, keratinocytes, and melanocytes by alternative immersion of the cell sheets. The dermal layer is made by seeding fibroblast on a flat surface that is immersed in cell culture media. Once matured, the sheet is raised and the second layer constituted of keratinocyte is added on top of the fibroblast layer. The multicell culture sheets are then immersed in a culture media for maturation of the cells by exposing the layers to the air–liquid interface. Developed in the 1990s, these skins cells are used for cosmetic testing. Indeed, since 2009 it is forbidden to use animals for cosmetic testing, and the artificial skins are now the industry standard for testing the safety of molecules used in cosmetic formulations. Current research aims at developing more advanced models including hair and nerve into the model.

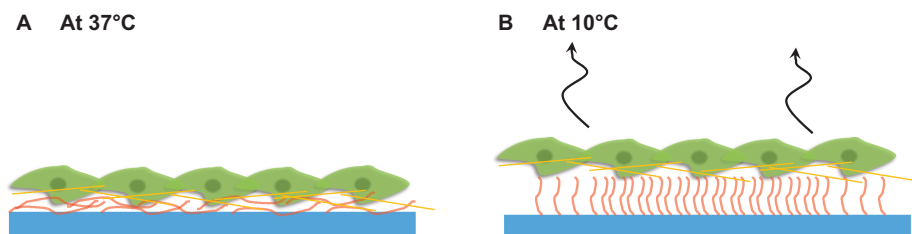


Figure 2.7: Temperature-actuated coating for cell sheet manufacturing. The petri dish is coated with poly(NIPAAm) polymer. At the cell incubation temperature (37 °C), the poly(NIPAAm) polymer forms a thin sheet coating. As the temperature is lowered, below the LCST, the polymer becomes hydrophilic and detached from the surface allowing to lift the cell layer with its ECM. The cell sheet can be detached and further used.



2.C Trypsinization

Once the cells have attached to the bottom of the tissue culture substrate, they will secrete macromolecules of the ECM and form strong bonds with the surface. To remove them, one needs to cut these bonds. The enzyme trypsin digests proteins by cutting the bonds between the amino acids. The coated proteins being cut becomes inefficient and the cells will detach and float in the media suspension. This process is called trypsinization.

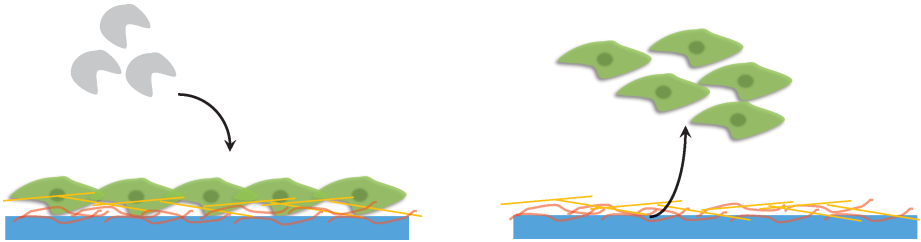


Figure 2.8: Action of the enzyme trypsin on the adsorbed molecules.

2.5 Biofabrication of spheroids

Spheroids are cells organized into spherical shapes. They are particularly interesting for the simulation of cancer tissues and cartilages [4]. To manufacture these spheroids, you need to force the cells to come together into a sphere. Naturally, cells will fall by gravity when suspended in a cell culture media, but not every cell type can form spheroids.

2.5.1 Hanging drop

Spontaneous aggregation into spheroids is a well-characterized and established methodology due to its ability to generate reproducible spheroids and their similarity to near-native tissue [5]. This methodology of spheroid aggregation has been exploited by the technique of hanging drop. In this method, a small droplet of cell suspension (single cell type or multiple cell type systems can be used) is pipetted onto the lid of a multiwell plate that due to the surface tension between the lid material and the water holds the droplet in place following its inversion (Figure 2.9). Density and type of cell suspension can be varied based on desired spheroid size or type. Following pipetting and as a function of time, the cells within the droplet accumulate at its tip, self-aggregate at the liquid-air interface, and eventually proliferate. During this technique, moisture/humidity within the plate is maintained to prevent drying of the droplet containing the cell suspension. This technique has the advantage of

being simple, inexpensive, and reproducible in terms of generating a single spheroid per droplet and results in tightly packed spheroids of cells.

2.5.2 Liquid overlay technique

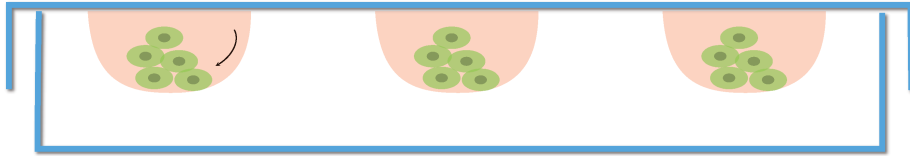


Figure 2.9: The hanging drop method is where individual cells are suspended in a droplet of media that is positioned on an inverted petri dish lid. The gravity forces the cells to come together into a spheroid.

As an alternative to the hanging drop methods, one can use a non-adhesive or attachment limiting substrate like agarose or non-adhesive petri dish to force cell aggregation. The cells added on these surfaces are forced to generate cell–cell attachment because they cannot generate cell–substrate attachment. This is a two-step technique: first, the cells are grown on non-adhesive tissue culture plates, wherein the cells migrate toward each other resulting in cell–cell aggregation and spheroid formation. In the second stage, the cellular aggregates grow. Several non-adhesive materials such as poly(2-hydroxyethyl methacrylate), polyethylene glycol, and agar can be used. While this is a simple technique, it suffers from several disadvantages including heterogeneity in size and cell number.

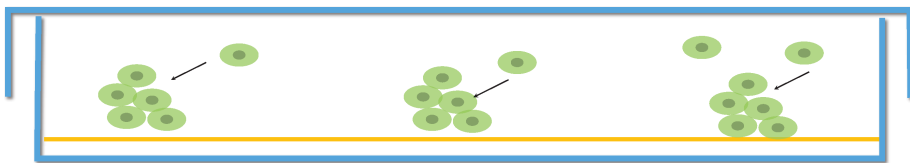


Figure 2.10: Using a non-adhesive coating (yellow), the cells cannot attach to the bottom of the cell and are forced to aggregate together.

In some cases, the coating can be a temperature-responsive material such as poly(NI-PAAM). As previously described, the cells can adhere to it and form a uniform sheet. When the temperature is lowered, the polymer solubilizes and the cells detach. Either the cells will stay together and form a sheet of cells or they will roll and aggregated into spheroids. This depends on the type of cells and incubation conditions.

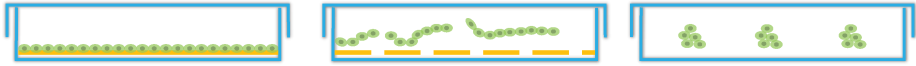


Figure 2.11: Temperature-sensitive poly(NIPAAm) forms an adhesive layer at 37 °C. At low temperature, the polymer solubilizes in water and the cells detached and aggregate into spheroids.

To obtain spheroids of homogeneous size, one can combine the coating technique with microwells that will limit the spheroid growth and size. For some cell types that naturally aggregate, microwells are sufficient to direct the formation of spheroids. But for some other cell types, it is challenging to induce the formation of spheroids. For instance, when the cell–cell attachment is not favored over the cell–substrate adhesion. In this case, non-adherent coating of the microwell is needed. This has the advantage to overcome the limitation of using only the non-adherent coating and enabling to control the spheroid size [6].



Figure 2.12: (left) Microwells that restrain the spheroid size and (right) same microwells that are coated with a non-adherent substrate that forces the cell aggregation.

2.5.3 Active rotational method

The methods presented above are classified as passive methods. But active methods are often needed to obtain spheroids reliably at a large scale. To this effect, spinner flask and rotary cell culture system (RCCS) are two of the agitation techniques used for growing cells in suspension culture (Figure 2.13). In the spinner flask, the cells are placed in a bioreactor that contains an impeller that maintains the cells in suspension. The cells then will come in contact with each other during the agitation and form spheroids. The motion provides a flux of nutrients as well as waste transport to and from the spheroids. The advantage of this technique is that it is easily scalable to the industrial scale of spheroid production, but one must take into consideration the shear forces generated by this technique which may alter the cell physiology. However, this method can result in the inconsistent size of spheroids [7].

The RCCS was introduced by NASA in 1992 to mimic microgravity [8]. This consists of a vessel that rotates on its horizontal axis and like in the spinner flask the constant rotation prevents the cells from adhering to the chamber walls. The culture vessel is filled with culture medium, inducing low fluid turbulence as well as low shear forces. Low fluid turbulence is provided in RCCS in comparison to the spinner flask as it reduce the shear forces induced to the cells.

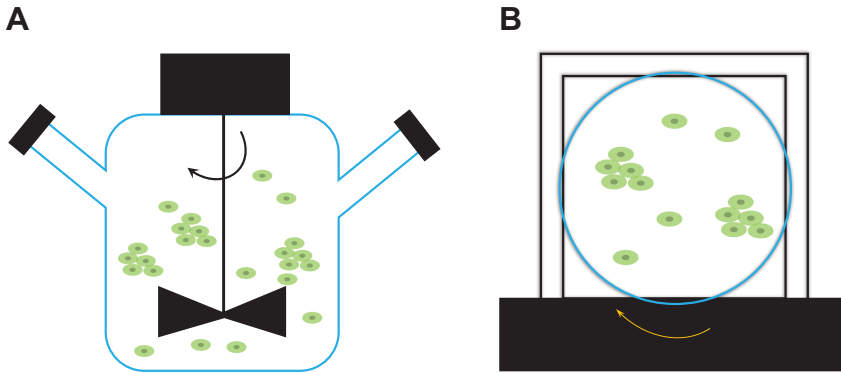


Figure 2.13: The two rotational techniques (**left**) are the spinning flask and (**right**) the rotary cell culture system (RCCS).

2.5.4 Microcarrier beads

Formation of cell aggregate can be obtained by centrifugation of a cell suspension in a Falcon tube or Eppendorf's tube. But this leads to a non-control spheroids size (Figure 2.14). Alternatively, microcarriers are spherical beads of diameter around $500\ \mu\text{m}$ that provide a defined surface area for the culture of a high density of cells in smaller volumes. This technique is particularly adapted for cells that do not aggregate spontaneously [9]. Microcarrier beads can be either solid or porous. Once the cells adhere, it is followed by cell growth. Some microbeads can degrade leading to the formation of a cellular aggregate. Non-degradable beads can also be used, but then the beads remain within the spheroid core. Microcarrier beads can also be coated with cell adhesion-promoting materials like gelatin, collagen, or laminin.

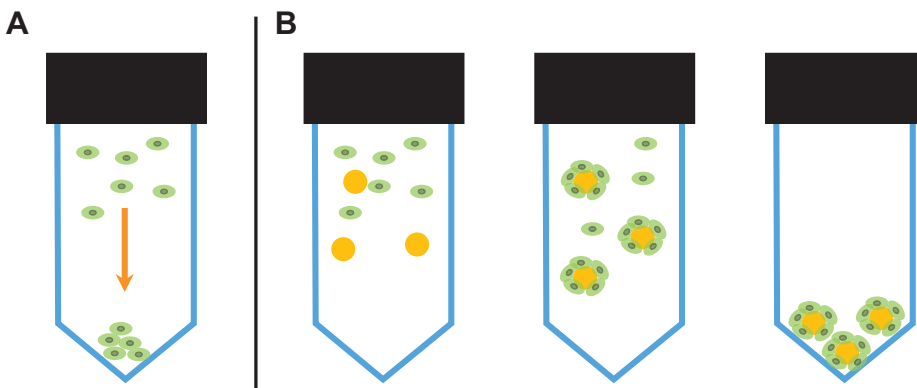


Figure 2.14: (**left**) Cells are forced to aggregate by centrifugal force. (**right**) Use of cell-adhesive microcarrier that aggregates the cells into spheres of equal size.



2.D Cell lineage

The cell lineage is the developmental history of a cell that can be traced back to the cell from which it comes. From the fertilized embryo, four main lineages are created which give the different cells observed in the adult organism. The current focus of research aims at isolating cells from abundant tissue that can be easily isolated such as fat and skin and manipulating the cells to create a different type of cells. However, biological limitations restrain the jump between different primary germ layers. To date, it is not possible to create a skin cell from a muscle cell (Figure 2.15).

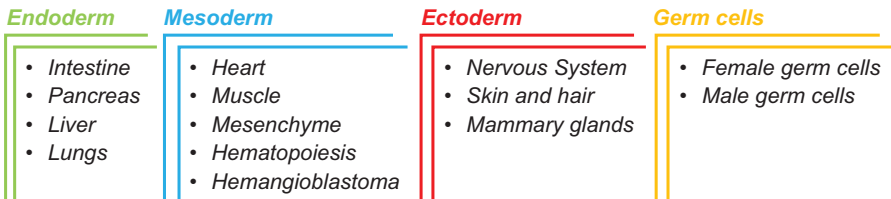


Figure 2.15: The four different cell lineage.

Mesenchymal stem cells are cells that can be harvested from adult individuals and differentiated into several types of cells of the mesoderm. They are isolated from different tissues including bone marrow, umbilical cord, and adipose tissues. Because mesenchymal stem cells can multiply without differentiation, these cells open the possibility to generate a high number of cells that can then be differentiated into a particular tissue (Figure 2.16).

Mesenchymal Stem Cells (MSCs)					
Cell	Osteoblast	Chondrocyte	Muscle cell	Epithelial cell	Adipocyte
tissue	bone	cartilage	muscle	skin	fat

Figure 2.16: The lineage of mesenchymal stem cells.

2.5.5 Magnetic levitation

In some cases, magnetic nanoparticles can be used to magnetize a cell population and then use magnetic force to manipulate the cells, Figure 2.17 [10]. This technique has been used for the generation of 3D tumor spheroids that are reminiscent of tumors *in vivo*. Usually, nanoparticles made of iron oxide (Fe₃O₄)-encapsulated in poly(lactic-co-glycolide) polymer are internalized by the cells, which become magnetic. Apart from the monoculture of cells using the magnetic levitation method, it has also been used for co-culturing to force cells of different origins to come together. While this technique utilizes magnetic nanoparticles that could potentially affect cell viability, the use of magnetic nanoparticles has been approved for imaging agents. Nevertheless, the

use of iron oxide as 3D spheroid models, in drug testing as well as tissue engineering, needs to be on a case-by-case toxicologically assessed.

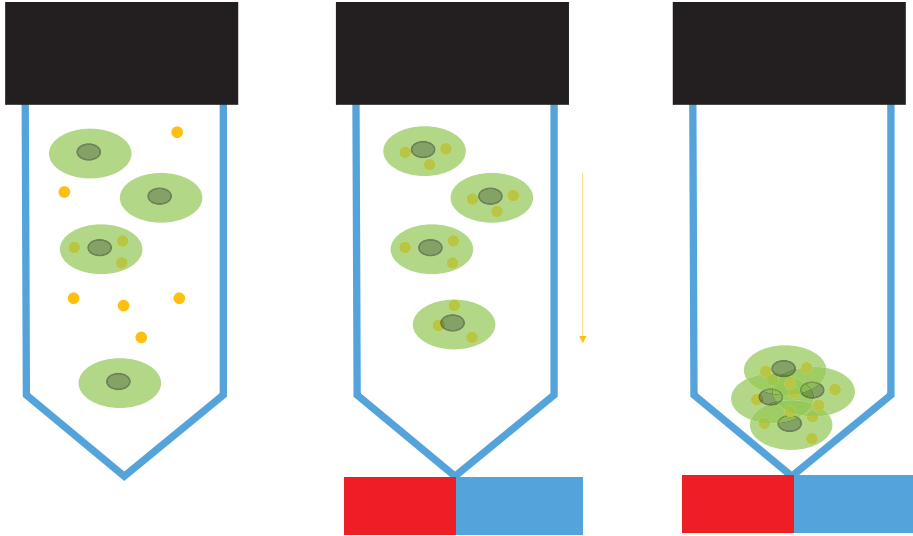


Figure 2.17: Magnetic nanoparticles (yellow) are incubated and uptaken by the cells. Upon exposure to a magnetic field, magnetized cells are attracted together to form a spheroid.

2.5.6 Acoustic aggregation

We have reviewed in the previous section that acoustic waves can be used to organize single cells into morphogenic structures. Similarly, spheroids can be created by forcing the cells to aggregate into gravity holes formed by an acoustic wave. Starting from a single cells suspension, an acoustic wave is generated that consists of peaks and depression in the aqueous culture media. Cells will then concentrate in the valleys and have the chance to aggregate into spheroids. Once these spheroids are created they can be further manipulated to form more complex structures using a different acoustic wave that will move the freshly created spheroids into a new pattern that will force the aggregation into tissue-like structures [11].

2.5.7 Emulsion

Emulsions are used in many industrial processes from polymer synthesis to food manufacturing. This technique uses the dispersion of water in oil and permits the creation of round structures that are isolated from each other. Applied to cell culture, cells can be dispersed into a water phase that is processed into an emulsion in

oil. Of course, in this application, the oils need to be non-cytotoxic and easily removable after the process to recover the cells. Cells are then encapsulated into the water phase and are forced to meet each other as they cannot escape into the oil phase. This emulsion creates several droplets like the hanging drop technique but in a process that can be scaled up in bioreactors. The water droplet size can be adjusted by optimizing the emulsion thus permitting the creation of homogeneous spheroids [12].

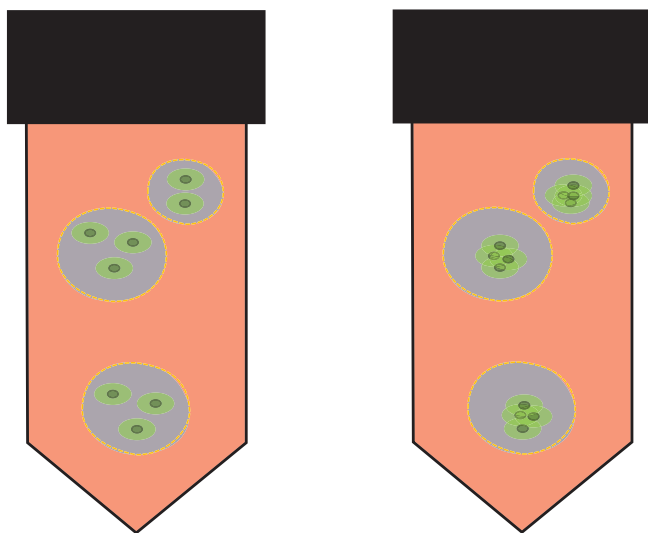


Figure 2.18: Emulsions of water (blue) in oil (red) are created which confines the cell in a set diameter.

2.5.8 Applications of spheroids

From the generation of organoids for drug testing to the formation of biological transplants, spheroids have a broad range of applications. One of the major challenges during drug development is to assess the liver toxicity of a drug. Therefore, being able to test the drug on a cellular organism that could accurately predict the human response *in vitro* would be of great benefit. Spheroid constituted of Kupffer cells, stellate cells, and biliary cells organized into spheroids have been shown to stimulate the effect of drugs on the liver [13]. In cancer studies, spheroids have played a critical role as they enable the reproduction of the necrotic center observed in tumors. The lack of oxygen in cancer tumors leads to the death of cells or a change in physiology. This can be reproduced *in vitro* using cancer tumor spheroids that are grown to a critical size [14]. Cancer cell spheroid when they reach a critical diameter, the core of the spheroid lacks of oxygen and becomes necrotic.

Apart from drug testing, spheroids can further be used as a building block to create a more complex or bigger tissue. For instance, spheroids are used as a building block for 3D printing using the needle array, called the Kenzan needles to form tubular structures. Spheroids are prepared in microwells and then strung on needles that are arranged in the shape of the attended tissue. For instance, the needles are organized into a cylinder and on each needle, spheroids are placed layer after layer creating first a circle and then a cylinder [15] (see Chapter on Bioprinting). The technique permits to rapidly create tissue with a high number of cells. The spheroids can be further manipulated using a 3D bioprinter that will position the spheroids into a particular shape. Using different spheroids complex tissues can be formed by the association of the organoids together. This technique was developed by Organovo and was attended to make liver replicates and other types of tissue to be used for drug testing and development.

2.6 Quiz

1. What is the major drawback of biomaterials for cell culture?
2. What is the ECM?
3. Why are spheroids important for cell structure?
4. Which additive manufacturing technique uses spheroids?
5. What are the four cell lineages?
6. What is trypsin and its role in cell culture?
7. What is the behavior of a polymer that has a LCST?
8. What is an example of polymer with LCST and one of its conditions applications?
9. Which spheroid manufacturing technique can simulate space conditions?
10. Which polymer can be used for a non-adherent coating?



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3 Organic and inorganic materials for biofabrication

3.1 What is a biomaterial?

A biomaterial is usually defined as a natural or synthetic material that is suitable for introduction into living tissue especially as part of a medical device. With this definition, we can conclude that biomaterials are non-cytotoxic and can be of different origins. But one must be careful on how to qualify a biomaterial. Indeed, a material can be biocompatible for a specific application but toxic for another application. The qualification is dependent on the usage and site of insertion. Following this principle, regulatory agencies do not qualify biomaterials for implantation, but medical devices made of biomaterials for a specific application. Hence comes the question of what sort of biomaterials can be used for biofabrication. One of the main uses of biomaterials in biofabrication is to make scaffolds that can be used to direct the organization of cells into tissues. So, biomaterials can be processed into scaffold, hydrogel, or surfaces that can be used as a cell substrate for cell culture. Biomaterials are classified into three major families: polymer, ceramic, and metal (figure 3.1). Each of these materials is used for implant or medical devices either as a degradable or non-degradable implant.

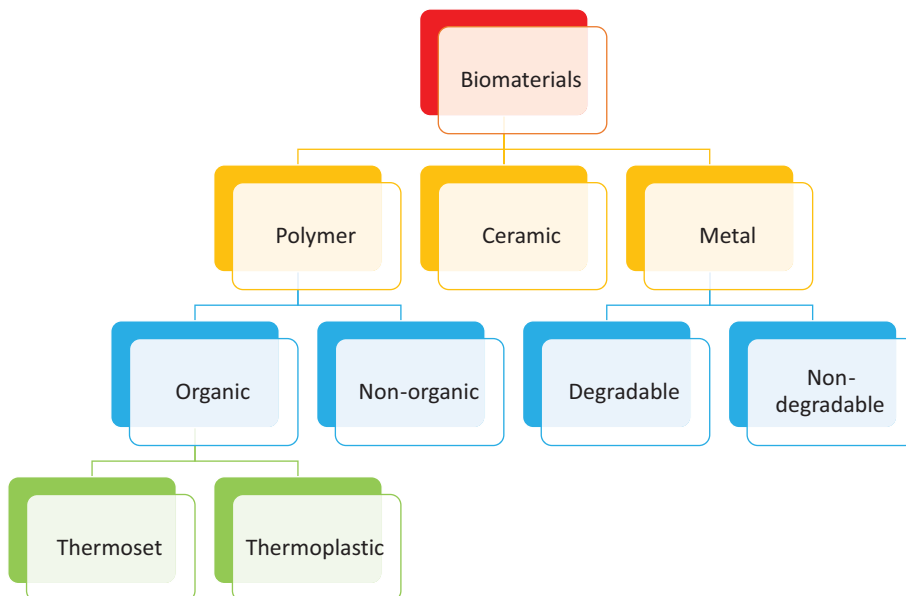


Figure 3.1: The subfamilies of biomaterials.

3.2 Organic polymers



3.A Polymer lexicon

Glass transition (T_g): The glass transition is the reversible transition in an amorphous or semi-crystalline polymer from a hard and brittle state into a viscous or rubbery state by increasing the temperature. The reverse transition, from a viscous liquid into the glass state, is called vitrification.

Melting temperature (T_m): The melting point of a polymer is the temperature at which it changes from solid state to liquid and all the chains are in a flow motion.

Thermoplastic: is a plastic polymer material that becomes pliable or moldable at a certain elevated temperature and solidifies upon cooling. As such, the polymer can be molded by pressure and heat. These materials are particularly suitable for mass production as they can precisely and rapidly process molded parts. Once molded the polymer part can be reproduced and sometimes recycles into other parts.

Thermoset: is a polymer that is obtained by the irreversibly hardening or curing of a soft polymer or viscous liquid prepolymer often called a resin. The resin or flowing polymer is introduced in a mold. Then, the curing, hardening, or setting of the polymer resin can be induced by heat or radiation or promoted by high pressure or mixing with a catalyst. Once the materials are cured, the thermoset polymers cannot be remolded.

3.2.1 Poly(styrene)

Poly(styrene) (PS) is an amorphous non-degradable thermoplastic with a glass transition (T_g) around 100 °C and a melting temperature of about 240 °C (figure 3.2). It is obtained by an anionic radical polymerization that allows getting high molecular weight polymer chains. Over the years, PS has become the material of choice for the culture of cells *in vitro*. It is used for making well plate and other plastic ware. It can undergo surface treatment in either chemical or plasma to modify its surface chemistry which in turn modulates the adhesion of cells. It can be manufactured at low cost, sterilized in bulk by gamma radiation, which permits to guarantee a sterile container at low cost for any cell experiment. Its good thermoplastic properties permit its molding, and it can be processed by 3D printing, thus opening new opportunities for prototypic the next generation of cell culture substrates.

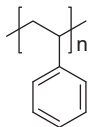


Figure 3.2: Chemical structure of poly(styrene).

3.B Anionic radical polymerization of styrene

The polymerization of styrene occurs through an anionic radical polymerization where two active sites are built on a chain and the polymer chain growth by both extremities (figure 3.3). (1.) The polymerization is activated with naphthalene, (2) the anionic radical is then transferred to the styrene monomer, and (3) the chain is grown, and the polymerization is propagated.

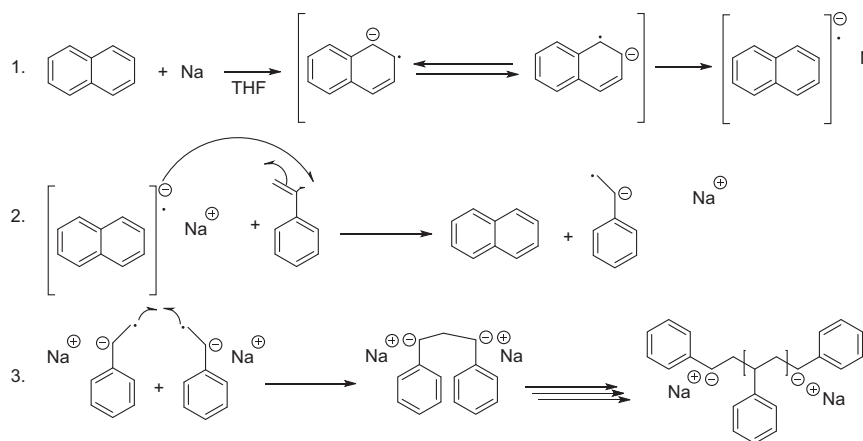


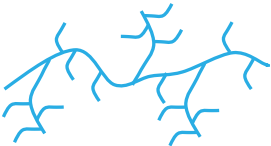
Figure 3.3: Radical anionic polymerization mechanism of poly(styrene).

3.2.2 Poly(ethylene)

Poly(ethylene) (PE) is the most abundant plastic material on earth. It is used for diverse applications from plastic bags to a container for biomedical applications including syringes and other medical devices. PE was originally synthesized by a German chemist, Hans von Pechmann, in 1898. But it was only after the Second World War that production ramped up with the discovery of new synthesis routes. The typical synthesis process for the polymerization of PE begins using ethylene, a gaseous hydrocarbon (C₂H₄) produced from petrochemical sources with a catalyst such as titanium (III) chloride, developed by Ziegler and Natta. PE obtained is semi-crystalline with a T_g around $-125\text{ }^{\circ}\text{C}$ for the low-density PE. It is a thermoplastic with a melting temperature ranging from $110\text{ }^{\circ}\text{C}$ to $140\text{ }^{\circ}\text{C}$ depending on the chain structure. Indeed, depending on the synthesis route one can obtain a partially branched polymer or linear polymer. The more the PE is linear, the higher will be the density. In addition to the branching, the length of the polymer chain can also vary depending on the catalyst used. There are therefore different types of PE polymer with different properties and domains of application. In medical devices such as implants, the ultra-high molecular weight high-density poly(ethylene) (UHMWPE) is mostly used (Table 3.1). It gives tough materials that are self-lubricant and is used

for the contact part for hip implants between the limb and the hip. Thus, UHMWPE acts as a load bearing replacement for articular cartilage.

Table 3.1: Reaction scheme of the free radical polymerization of poly(ethylene) and a table summarizing the different types of poly(ethylene) and their polymer chain structures. I being a radical initiator.

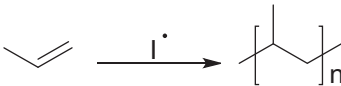
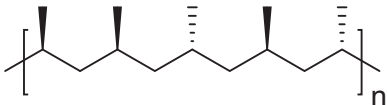
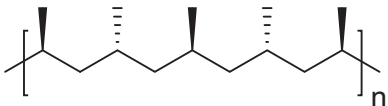
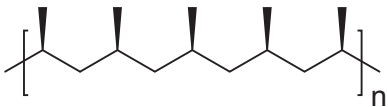
$\text{CH}_2=\text{CH}_2 \xrightarrow{\text{I}^\cdot} \left[\text{CH}_2-\text{CH}_2 \right]_n$			
Type of PE	Name	Density	Chain
Low density	LDPE	0.88–0.91	
Linear low density	LLDPE	0.91–0.925	
Medium density	MDPE	0.926–0.94	
High density	HDPE	0.941–0.965	
Ultra-high molecular weight	UHMWPE	>0.966	

3.2.3 Poly(propylene), (PP)

PP is a semi-crystalline thermoplastic with a glass transition around 0 °C and a melting transition ranging from 130 °C to 171 °C depending on the crystallinity degree. Because of the methyl side chain, PP can only be synthesized with metal catalysts such as Ziegler–Natta. The catalyst permits the control of the molecular structure of the obtained materials and the orientation of the methyl group on the chiral carbon. Indeed the methyl group of the PP can be positioned in a different position depending on the monomer incorporation in the polymer chain. For methyl that is all on the same side, it is an isotactic (all chiral carbons are R or S) PP. If there is alternating R and S chiral carbon, the PP is syndiotactic, and if there is no organization, the PP is atactic Table 3.2. PP is used in many biomedical applications for making non-degradable implants such as hernia mesh, but it is

often associated with a foreign-body response. Due to PP good mechanical properties, it is used for sutures and has a mesh to maintain the organ in place after trauma or surgery. It is also used to make many devices such as syringes and other medical containers used for holding solutions for injection.

Table 3.2: Reaction scheme of the free radical polymerization of poly(propylene). I being a radical initiator.

				
Molecular structure	Type	Crystallinity	T _g	T _m
	Atactic	Amorphous	-20 °C	0 °C
	Syndiotactic	Semi-crystalline	-8 °C	160 °C
	Isotactic	Semi-crystalline	0 °C	184 °C

3.C Ziegler–Natta catalyst

Ziegler–Natta mechanism for the polymerization of poly(propylene) (PP) and high molecular weight PE (figure 3.4). Where L is an unspecified ligand, M is a metal such as Al, Li, Mg, or Zn and R is an alkane chain. First, the catalyst is activated and coordinated with an alkene monomer. A rearrangement occurs and the chain starts to grow. The coordination rearrangement is repeated as many times as the monomer is integrated into the polymer.



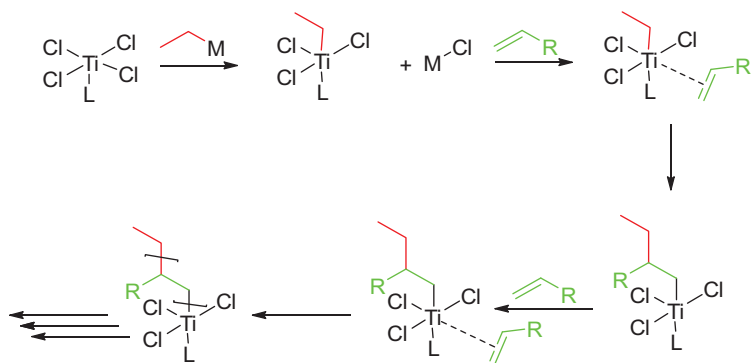


Figure 3.4: Ziegler–Natta mechanism.

3.2.4 Poly(tetrafluoroethylene)

Poly(tetrafluoroethylene) PTFE is a semi-crystalline polymer with a T_g of 115 °C and a melting temperature T_m of 327 °C (figure 3.5). However, the melting temperature is close to its degradation temperature, and it is therefore challenging to process it in the melted state. The polymer is obtained by radical chain polymerization from the gaseous monomer tetrafluoroethylene. Due to its fluorine atoms, PTFE has low friction, is anti-fouling, and is highly hydrophobic which makes it a material of choice for certain biomedical applications. For example, membrane filtration, as it permits to avoid adhesion of the molecules being filtrated. But the exceptional thermo-mechanical character of PTFE (thermal isolation and low flow) makes it hard to process like the other thermoplastics and so PTFE is usually machined to create parts. However, because it releases small debris, it cannot be used for low friction applications like for articular joints. For these applications, UHMWPE is preferred. The expanded form of PTFE (Gore-Tex™) is used for vascular prostheses, sutures, and some cardiac patches. The expansion process makes the materials porous, waterproof but allow for the exchange of gas.



Figure 3.5: Chemical structure of poly(tetrafluoroethylene).

3.2.5 Poly(amide), Nylon

Nylon is usually obtained by polycondensation of sebacoyl chloride with diamine monomers [1]. It is a semi-crystalline material with a T_g of about 130 °C and a melting temperature from 178 °C to 265 °C depending on the length of the alkane chain (figure 3.6). Because amine groups can form interchain h-bonds with the oxygen atoms, polyamide (PA) forms naturally long strands of materials that are easily processed as a fiber. Therefore, one of the main applications in the biomedical field for nylon was found for sutures. Another application for PA was for the manufacturing of balloon catheters as it provides a material with a high strength that allows the balloon to be bloated with high pressure.

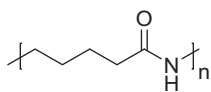


Figure 3.6: Chemical structure of poly(amide).

3.2.6 Poly(carbonate)

Poly(carbonate) (PC) is an amorphous polymer with a T_g of 147 °C and with a melting temperature between 230 °C and 260 °C (figure 3.7). PC is obtained by polycondensation of bisphenol A (BPA) and phosgene. However, due to safety concerns, intensive research is focusing on finding solutions to remove the use of BPA. That is BPA is reported to have hormone-mimicking properties and is therefore said to be a chemical to which humans should not be exposed. Because of its amorphous properties, PC gives a transparent material. This property is quite important for many applications where the samples need to be monitored in a container. For instance, when handling blood samples, monitoring flow, color, and coagulation.

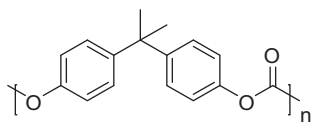


Figure 3.7: Chemical structure of poly(carbonate).

3.2.7 Poly(vinyl chloride)

Poly(vinyl chloride) (PVC) is the third most-produced polymer with a T_g of 82 °C and melting temperature of 260 °C; PVC is usually a rigid material at room temperature (figure 3.8). However, by the addition of plasticizers such as phthalate, citrate adipates,

or azelates the plasticity of PVC can be tuned [2]. Its low cost and ease of manufacturing makes it a candidate of choice for the cost-effective manufacturing of high-volume devices and their packaging. Additionally, PVC can be sterilized with many methods from autoclave, ethylene oxide, and chemical sterilization processes. But the main concern of PVC is the addition of plasticizers that could be leaking from the bulk materials and contaminate the surrounding environment. In cause, the phthalate, which after many studies over the past decades, there is to date a lack of data showing harmful effects of phthalate that would prevent the use of PVC for medical devices. About one-third of disposable medical devices are manufactured from flexible PVC. It is mostly used in medical devices that interact, both directly and indirectly, with human body fluids or tissues (e.g., blood or intravenous storage bags and/or tubing). It is widely employed in the storage of blood derivatives.

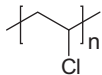


Figure 3.8: Chemical structure of poly(vinyl chloride)

3.2.8 Poly(ethyl vinyl acetate)

Poly(ethyl vinyl acetate) (PEVA or EVA) is a copolymer that is obtained by the polymerization of vinyl acetate with ethylene in any proportion (figure 3.9). It is a semi-crystalline thermoplastic. The ratio of each monomer will affect the properties and thus resulting in diverse applications for the copolymer. An increased proportion of vinyl acetate in the copolymer induces an elastomer type of behavior which is favored for providing resistance to shock, transparency, and gas permeability. These enhanced properties are achieved to the detriment of the resistance to traction, heat, and melting point. Typical PEVA will have a T_g of about $-25\text{ }^{\circ}\text{C}$, and a melting temperature between $45\text{ }^{\circ}\text{C}$ and $105\text{ }^{\circ}\text{C}$ depending on the content of vinyl acetate [3]. EVA at industrial scale is usually polymerized in bulk, through an exothermic process above $100\text{ }^{\circ}\text{C}$ under pressure. But medical-grade polymerization occurs in solution at low pressure and usually requires the use of a buffer to maintain the pH and reduce the hydrolysis of acetate. The main use of PEVA is for plastic packaging for food and agricultural application as it provides an adhesive film due to the capabilities of the acetate moieties to adhere to polar substrates such as paper. PEVA is used in several controlled drug delivery devices for small and large molecular weight drugs. These devices are fabricated by using casting and freeze-drying methods and are mainly used for subcutaneous implantation. Varying the content of vinyl acetate copolymer in the bulk affects the permeability of the device and thus the delivery profile of the hormone. One of the major successes of PEVA implants is the estrogen delivery rod that permits a sustained delivery and contraceptive effect for up to 3 years. The low melting temperature and the possibility to mix it with active pharmaceutical ingredient makes

PEVA a great candidate for biofabrication applications as it can be processed by 3D printers [4, 5].

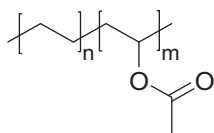


Figure 3.9: Chemical structure of the copolymer poly(ethylene vinyl acetate). Adjusting the ratio of vinyl acetate can be used to precisely tune the properties of the resulting polymer.

Drug release mechanism

There are three main mechanisms of drug release for a drug carrier (figure 3.10). The carrier loaded with its payload will release its cargo by diffusion of the molecule through its polymer network from the inside to the outside. This is the main mechanism in the hydrogel type of carrier. In a bioresorbable polymer system, the payload will be released as the carrier degrades, and here there are two main degradation mechanisms. These polymers are degrading through aqueous hydrolysis. So, the type of degradation erosion of the polymer will be dictated by the ratio between water diffusion and degradation rate of the polymer. If the water diffuses to the polymer bulk faster than its degradation, then the carrier will have a bulk degradation or erosion leading to a porous material. If the polymers degrade faster than the water can diffuse in the polymer then the carrier will have surface erosion, here only the surface of the materials is degraded. In turn, we will have two different release

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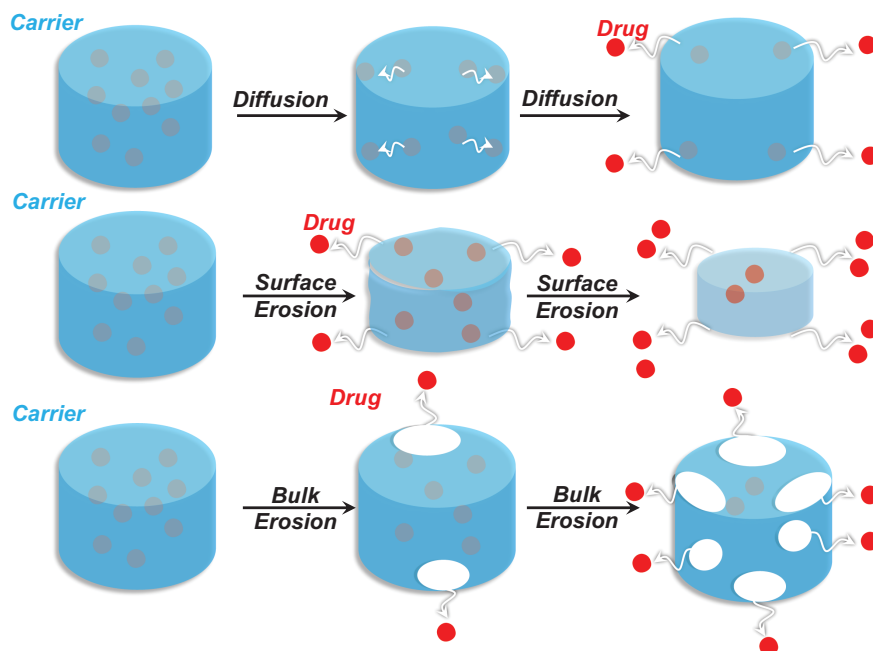


Figure 3.10: The three different drug release mechanisms of drug-loaded polymer are: diffusion of the active pharmaceutical ingredient out of the polymer matrix, erosion of the polymer matrix from the surface, and erosion of the bulk polymer matrix.

kinetic profiles. A surface erosion rate will give a near-zero-order kinetic release and the release will be directly proportionate to the surface area of the materials. Conversely, bulk erosion is more difficult to control, and a more complex kinetic order is achieved. Here the degradation rate is proportional to the volume of the implant and so as the implant erodes, its volume declines and its release rate as well. Therefore, the materials for the drug delivery system need to be meticulously chosen for each drug delivery application. Typically, polyester polymers such as polycaprolactone (PCL), poly(lactic acid) (PLA), and polyglycolic acid (PGA) undergo bulk erosion. Polyanhydride and poly(orthoester) are conversely, considered as surface eroding [31].

3.2.9 Poly(ether ether ketone)

Poly(ether ether ketone) (PEEK) is a polymer parts of the larger family of poly(aryl ether ketones) that comprise several variations of the ether ether ketones and ether ketone ketone polymeric architecture (figure 3.11). PEEK is a semi-crystalline amorphous polymer with a glass transition that occurs around 143 °C and a melting temperature around 345 °C. Its high-performance mechanical properties exhibit an elastic modulus up to 4 GPa and can be reinforced by different phases such as glass fibers, hydroxyapatite, and metallic oxides to match bone elastic modulus (18 GPa). The material is obtained by a step-growth polymerization process through the reaction of 4, 4 difluorobenzophenone with hydroquinone sodium salt at temperatures above 300 °C in aprotic solvents (Figure. 3.11). PEEK is a biocompatible material that does not degrade *in vivo*, it is thus a great material for prosthetics implants to replace even load-bearing bone defects. Although the melting temperature of PEEK is extremely high, it can be processed by a high-performance 3D printer capable of reaching a temperature above 390 °C, above the flow temperature of the polymer. This permits the creation of custom-made polymeric implants for reconstructive surgery of bones [6, 7].

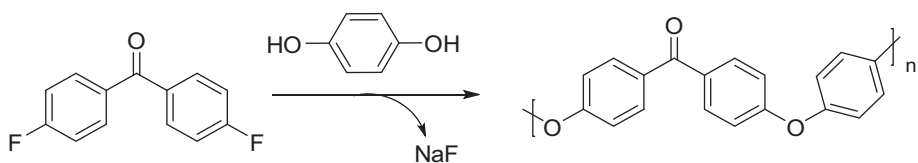


Figure 3.11: Schematic of the chemical reaction for the step-growth polymerization of poly(ether ether ketone) polymers.

3.2.10 Poly(glycolic acid)

Poly(glycolic acid), (PGA) is a thermoplastic semi-crystalline polymer that has a glass temperature of 40 °C and a melting temperature of 230 °C. This material is used to make biodegradable surgical sutures (figure 3.12). PGA undergoes biodegradation

through hydrolysis of the ester bond between the repeating units of the polymer chain. This degradation is catalyzed by an alkaline and acidic environment. This material can also be processed as a mesh for wound dressing. Because it is thermoplastic, it is suitable for its processing through melt extrusion 3D printing. PGA can be synthesized through different polymerization routes, but the most efficient one is through ring-opening polymerization (ROP) of glycolide catalyzed by tin octoate, a catalyst approved in polymer used in food and medical application (Figure. 3.12). The polymerization of lactone by $\text{Sn}(\text{Oct})_2$ is undergone through a coordination–insertion mechanism. The polymer obtained through ROP is of high molecular weight ($M_n > 100 \text{ k g.mol}^{-1}$) and high crystallinity of about 50%. PGA is considered a hydrophilic polymer. Due to its good wettability, its degradation rate is relatively fast. For instance, *in-vivo* PGA can lose about 50% of its weight in a couple of weeks. The addition of a co-monomer through polycondensation allows for the precise tuning of the physical properties of the polymers such as degradation rate, thermal and mechanical properties. Because of its thermoplastic properties, PGA can be processed by fuse deposition modeling 3D printing to make biodegradable scaffolds [8, 9].

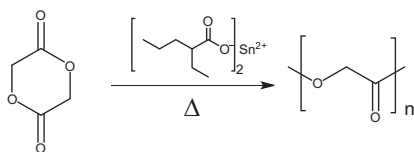


Figure 3.12: Ring-opening polymerization of glycolide to obtain PGA using tin(II) catalyst.

3.2.11 Poly(lactic acid)

Poly(lactic acid), (PLA) is a thermoplastic semi-crystalline polymer that has a glass transition at 60 °C and a melting temperature of 150 °C (figure 3.13). However, it can be processed into a transparent amorphous film by casting the PLA in a cold mold without a nucleation agent (Figure 3.13). PLA is a fully biobased polymer that is obtained by ROP of lactide extracted from corn starch. The starch is depolymerized through acid or enzymatic treatment to obtain glucose. The glucose undergoes then glycolysis through an enzyme treatment to give lactide. The process is achieved by fermentation of the starch using microorganisms such as the *Lactobacillus* bacteria. The fermentation process dates back to the nineteenth century, but with the recent advances in biotechnology, the process has been improved to obtain biobased-monomers with cost competing with oil-based monomers. Once the lactide is obtained, it is polymerized through a ROP using tin octanoate following the same mechanism as for PGA. In contrast to PGA, PLA has asymmetrical methyl groups which convey its hydrophilic properties and longer degradation rate. However, both lactide and glycolide monomers can be polymerized following the same process, they can then be polymerized together to form a copolymer poly(lactic-co-glycolic acid) (PLGA).

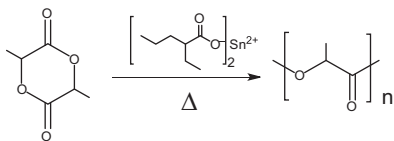


Figure 3.13: Synthesis of poly(lactic acid) from lactide monomer using heat with the tin(II) octanoate catalyst.

3.2.12 Poly(lactic-co-glycolic acid)

Poly(lactic-co-glycolic acid), (PLGA) is also a thermoplastic copolymer with a melting temperature of about 250 °C and glass temperature above physiological temperature, usually about 40 °C (figure 3.14). Controlling the ratio of PGA and PLA in the PLGA copolymer allows for the precise optimisation of the degradation rate of the polymer and its melting and hydrophobic properties. Because PLA contains an asymmetric methyl group, PLA is more hydrophobic than PGA. Therefore, a higher content of PLA in the copolymer will increase its hydrophobicity. The increased hydrophobicity will in turn decrease the interaction of the polymer with the aqueous milieu and reduce its degradation rate. For PLGA, the degradation rate can be controlled by two mechanisms: the molecular weight of each block of the polymer and the ratio of PGA to PLA (Figure 3.14). The possibility to precisely control the degradation rates of PLGA permits designing drug release systems in which the cargo is released at a constant rate. The drug is dispersed into the polymer matrix, the polymers degrade thus releasing the drug. The PLGA copolymer can be prepared as a block copolymer or as a random copolymer. The block copolymer allows forming micelles or liposomes nanoparticles whereas the random copolymer is more suited for the manufacturing of eluting implants and microparticles. The PLGA is used for drug delivery in the oral and parenteral formulation. Alternative synthesis routes of PLGA are being investigated to remove the need for a tin catalyst, despite the tin octoate being approved in polymer used in food and certain biomedical applications.

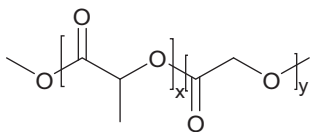


Figure 3.14: The chemical structure of poly(lactic-co-glycolic acid) shows the block copolymer structures that allow to precisely tune the hydrophobicity and degradation rate of the polymer by changing the ratio of lactic acid to glycolic acid.

3.2.13 Polycaprolactone

Polycaprolactone, (PCL) is a thermoplastic polymer with a glass transition of -60 °C and depending on its degree of crystallinity a melting temperature ranging from 59 °C to 64 °C (figure 3.15). Due to its degradation by hydrolysis and ease of manufacturing, PCL has been for a long time a polymer of choice for engineering medical devices.

Due to its low melting point, it is particularly suitable for advanced manufacturing techniques such as 3D printing and melt electrowriting, further described later in this book. High molecular weight PCL is usually achieved by ROP of ϵ -caprolactone. Several catalysts can be used for the polymerization of PCL including metal-based, organic, inorganic, and enzymatic systems. While metal-based catalysts are predominantly used, enzymatic systems are being intensively studied. The catalyst is mainly chosen for an application and the desired reaction conditions. For biomedical applications, the tin octanoate catalyst is mainly used due to its history in food and biomedical space. PCL degrades slower than PLA, PGA, and copolymer thereof. up to several years for PCL to totally degrades *in vivo*. The addition of copolymer can modify the degradation profile of the resulting materials and thus allow for the design of drug delivery devices with degradation profiles adapted to a specific application. In the human body, degradation is a two-step process. During the first year, ester groups are hydrolytically cleaved. Next, intracellular degradation occurs in the second phase beginning when the polymer is more highly crystalline and of low molecular weight, typically below 3,000 g. mol⁻¹. At this stage, different cells can degrade in about two weeks the fragments such as macrophage and giant cells. But the degradation is ultimately dependent on the molecular weight and crystallinity of the polymer or composite.

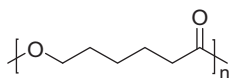


Figure 3.15: Polycaprolactone is polymerized through ring-opening polymerization of ϵ -caprolactone. Several polymerization strategies are available including enzymatic, anionic, and cationic.

3.D Coordination–insertion mechanism for ring opening polymerization (ROP)

Many polymers discussed in this chapter are synthesized by ROP. The most common mechanism for the ROP is the catalyzed coordination–insertion mechanism with tin octanoate (figure 3.16). Here, the metal catalyst coordinates with the lactone to open the ring and insert one of its alkoxy groups. Here, the oxygens are colored to highlight the mechanism.

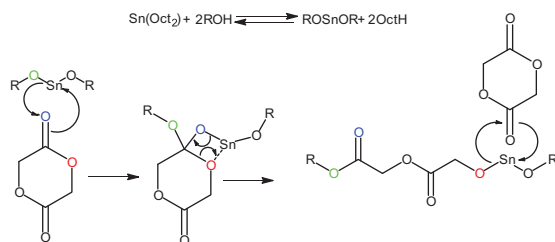


Figure 3.16: Ring-opening polymerization mechanism.

3.2.14 Poly(vinyl alcohol)

Poly(vinyl alcohol) (PVA) is a semi-crystalline water-soluble polymer with a glass transition of 80 °C and a melting temperature of 200 °C (Figure 3.17). Because the monomer, vinyl alcohol, is thermodynamically unstable due to its tautomerization to acetaldehyde, PVA cannot be directly synthesized by radical polymerization. PVA is obtained by hydrolysis of poly(vinyl acetate), through a base-catalyzed transesterification with ethanol. Because of its alcohol functional groups, PVA can be processed as a physical hydrogel through the formation of complexes with divalent cations such as calcium or copper, and as chemical hydrogels using crosslinkers such as boric acid, glutaraldehyde (see Chapter 4 for hydrogels). PVA hydrogel can be obtained by the crystallization of the polymer in a DMSO/water solution which upon cooling below room temperature will induce the crystallization of the polymer and its crosslinking. Exchanging the DMSO solvent with water gives a transmitting hydrogel of high tensile strength. One of the applications of PVA chemically crosslinked hydrogel is used to make degradable contact lenses. PVA is completely degradable by microorganisms and the mechanism involved enzymatic dehydrogenases and secondary alcohol oxidases. But the main application of PVA is to form fibers for the textile industry and as an additive, in many formulations of composites and cement as they can serve as a hydrating phase.

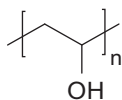


Figure 3.17: Chemical structure of the biodegradable thermoplastic PVA. Obtained through the saponification of polyacetate.

3.2.15 Polyanhydride

Polyanhydrides were first investigated by Hill and Carothers at the beginning of the twentieth century, but their low aqueous stability is a great limitation for their industrial application (Figure 3.18). With the development of tissue engineering and drug delivery system, their degradation behavior became of interest for sutures and the engineering of diverse medical devices. Polyanhydride is synthesized by dehydrating carboxylic acid difunctional monomers to form corresponding polyanhydride linkages. The usual industrial route involves the use of sebacic chloride to reach a typical molecular weight of about 15–20 kDa. Like any anhydride functional group, the polymer chain is highly sensitive to moisture and quickly degrades in water through a hydrolysis mechanism to give the original carboxylic acid monomers. Because sebacic acid naturally occurs in human physiology, it is a good monomer to use

in a biodegradable polymer. Like the other polymer systems presented earlier, their degradability rate can be tuned by introducing hydrophobic groups such as aromatics which will reduce the water access to the polymer chain and thus the degradation rate. Additionally, their molecular weight and degree of crystallinity will affect their degradation rate, the higher the crystallinity and the higher the molecular weight, the slower the polymer will degrade. Typical degradation occurs in a couple of weeks. Depending on the monomers polyanhydride will have different glass transition temperatures (T_g). For instance, an aromatic repeat unit will give a polymer with a T_g above physiological temperature while having a solubility in organic solvent, and a melting temperature typically between 50 °C and 90 °C. Thus, these thermochemical properties make it a polymer easy to process.

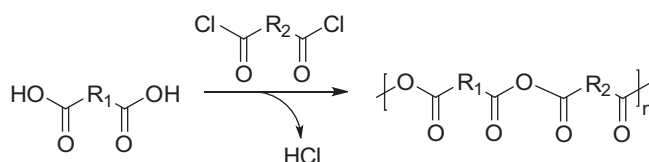


Figure 3.18: Polymerization of a polyanhydride by the reaction of a dicarboxylic acid with diacyl chloride monomers. The polymer quickly degrades in the presence of water.

3.2.16 Poly(propylene fumarate)

Poly(propylene fumarate) (PPF) is an amorphous thermoplastic polymer with T_g dependent on the molecular weight and is typically below physiological temperature (10–30 °C) (Figure 3.19). It is usually synthesized in an organic solvent by step-growth polymerization using the various metal catalyst available including chromium (III) complex, or manganese dietanoate from maleic anhydride and propylene oxide. Other synthetic routes involve a multistep method using diethyl fumarate with propylene oxide and a catalyst such as zinc chloride. The interesting properties of PPF are that it can be crosslinked by radical polymerization using a UV photoinitiator. This has particular interest for the manufacturing of bone or dental implants as the polymer can be molded into the defect space and then harden using UV crosslinking. Additional crosslinking molecules can be used such as poly(ethylene glycol) terminated with methyl acrylate, for which the molecular weight can be varied to the obtained resin of diverse mechanical properties up to the range of MPa elastic modulus reproducing the toughness of bones. The resin is then degraded through hydrolysis, and it is highly impacted by the molecular weight of the polymer, its crosslinking density, and crosslinker type. As we have reviewed for the previous polymers, higher molecular weight and denser resin will decrease the degradation rate [10, 11].

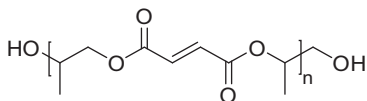


Figure 3.19: The chemical structure of poly(propylene fumarate) PPF can be further crosslinked by radical polymerization into a resin due to its unsaturated carbon bond.

3.2.17 Polyurethane

Polyurethane (PU) is synthesized by step-growth polymerization to obtain a semi-crystalline polymer. PU is an interesting polymer that can be obtained as foam, thermoset materials but also thermoplastic materials. These differences can be obtained by making either homopolymer (thermoset) or block copolymer made of segments that contain long linear blocks made of hard and soft segments. The soft segments are built with polyols that provide flexibility to the polymer chains and the hard segments are built with diisocyanate monomers that are not flexible (Figure 3.20). As a thermoset, PU can be processed as foam either with close cells or open cells geometry. In an open-cell system, the pores created by the foaming reaction are interconnected, this is usually generated by the addition of extra gas. Whereas in a close cell system, the pore generated by the foaming process is not interconnected. For biomedical applications and implants, it is generally preferred to have open cells where cells can penetrate and migrate within the implant. Once the implant is colonized by the cells, they can generate their matrix and create artificial tissue. PU can undergo degradation *in vivo* through the hydrolysis of the urethane bonds. In thermoplastic PU, the degradation usually occurs at the intersection of hard and soft segments [12].

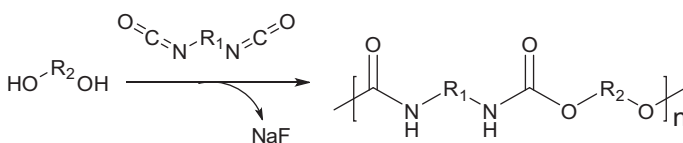


Figure 3.20: Scheme of the chemical reaction for the step-growth polymerization of the polyurethane.



3. C Immune response cells fouling on biomaterials

The response of a material once implanted is a combination of several factors: its surface chemistry, pore shape and size, and surface roughness. In this chapter, we have reviewed different materials which have diverse chemical properties. Once implanted in tissue, proteins from the surrounding tissues will adhere differently depending on the chemical nature of the polymer surface (Figure 3.21). The chemistry of the polymer: hydrophobic, charged, or hydrophilic will help select the type of molecules that adhere onto the surface and in the case of protein the chemistry

can also alter their folding on the surface. In turn, cells from the immune systems will experience different types of molecules and molecule folding at the polymer surface. As a result, different types of cells will then adhere to the surface. Alternatively, the same progenitor cells could adhere to the surface but depending on the information obtained on the polymer surface will differentiate into two distinct cell lineages and secrete different chemokine which will, in turn, convey a signal of “isolation” or “integration” of the implanted materials. We can see by this mechanism that selecting the right polymer for a specific application is paramount for the good integration or isolation of the implanted material. Some applications require the implant to stick with the tissue to have integration such as implants used for the replacement of load-bearing bones. But other applications such as drug delivery rods that need to be removed once the cargo is delivered after several years, the polymer cannot be integrated into the tissue as it would limit the removal of the implant.

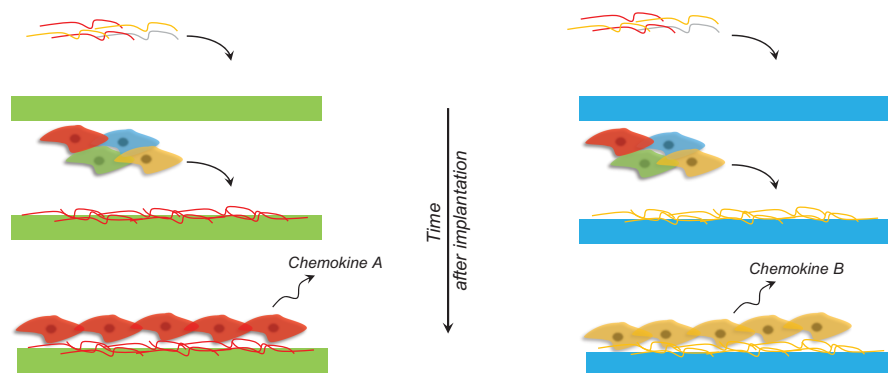


Figure 3.21: Comparison of implant behavior after implantation. The green material adsorbs the red protein, which in turn recruits the red cell that will set a biological signal using a chemokine. Conversely, the blue material adsorbs the yellow protein which recruits the yellow cell that will send a different signal. Thus, green and blue materials will not induce the same biological response after implantation.

3.2.18 Poly(hydroxy alkanates)

Poly(hydroxy alkanates) (PHAs) are semi-crystalline thermoplastic polymers with a T_g varying between $-50\text{ }^{\circ}\text{C}$ and $5\text{ }^{\circ}\text{C}$ and a melting temperature of comprised between $60\text{ }^{\circ}\text{C}$ and $180\text{ }^{\circ}\text{C}$ depending on their molecular structure (Figure 3.22). These polymers are obtained by bacteria biosynthesis and are used by the bacteria as energy storage materials. The longer the side chain R is the higher is the T_g and T_m . As a polyester, PHA can be degraded by hydrolysis but also by bacterial depolymerase. The PHA usually degrades in 4 years *in vitro* under physiological conditions. Due to their tunable properties, these materials have been used for the biofabrication of a broad range of soft and hard tissue by changing the R side chain chemistry. Other thermoplastic PHAs can be formulated for drug delivery applications and be processed as fibers and

microparticles. Due to their slow degradation rates, the drug delivery applications are limited to long-term ones. But they still suffer from several drawbacks mainly the production cost and the melting, the melting temperature is often close to the degradation temperature. The current use of PHA is directed toward blends with other natural organic polymers. Finally, the modification of the polymer required genetic engineering of the bacteria, and this is an expensive and long process. Additionally, the needed to obtain pure materials are tedious and costly as for biomedical application one needs to remove pyrogenic materials issue from the bacteria wall because lipopolysaccharides can cause inflammation once implanted [13–15].

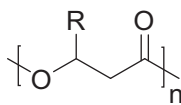


Figure 3.22: Scheme of the chemical structure of poly(hydroxy alkanate) polymers and their nomenclature.

	Alkyl (R)	Name Poly(3hydroxy-)	Abb.
	H	Propionate	PHP
	CH ₃	Butyrate	P3HB
	CH ₂ CH ₃	Valerate	PHV
	Propyl	Hexanoate	PHHx
	Butyl	Heptanoate	PHH
	Pentyl	Octanoate	PHO
	Hexyl	Nonanoate	PHN

3.2.19 Poly(ether sulfone)

Poly(ether sulfone) (PES) is an amorphous non-degradable thermoplastic polymer that has a glass transition around 188 °C and can be sterilized by many processes, which makes it a good candidate for biomedical applications. These polymers can be made either by polycondensation or ROP (Figure 3.23). The resulting polymer is transparent and can be used for medical devices that can withstand many steam sterilization cycles without deformation. Thus, it can be used to make surgical tools as an alternative to stainless steel. PES starts to flow at a temperature above 340 °C but still can be processed by a high-performance 3D printer. It can be made as a composite with an inorganic calcium phosphate phase for dental implants.

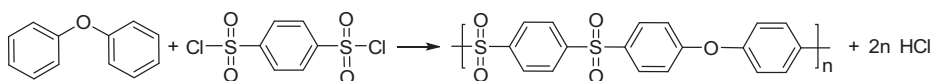


Figure 3.23: Scheme of the polymerization reaction of poly(ether sulfone).

3.3 Synthetic inorganic biomaterials

According to IUPAC, an inorganic compound is defined by a compound that is not organic. An organic molecule is a molecule that contains carbon bound to hydrogen. As such carbon dioxide is not only an organic molecule. Counter-intuitively to the popular belief that living objects are composed of organic molecules, mammals are predominantly composed of inorganic materials. Indeed, the mineral phase in mammalian tissue accounts for about 70% of the whole body. While the organic phase is composed of the majority of cells and extracellular matrix (collagen being the most abundant polymer in the tissues), the bones, teeth, and salts found in the aqueous phase (Mg, Na, K, Ca, F, and Cl) account for most of the total body mass.

3.3.1 Calcium phosphate

The inorganic phase of the bone is composed of a calcium phosphate crystal that has a specific Ca/P ratio of 1.67 called hydroxyapatite (HAp). But reproducing this exact ratio *in vitro* and at an industrial scale is quite challenging. Indeed, several calcium phosphate crystals can be generated. Some of them reproduce naturally occurring crystals. Each different ratio of calcium to phosphate leads to a different crystal structure that has then different biological activity (Table 3.3). These materials are used to support the regeneration of bones by providing a natural environment for osteointegration. The HAp can also be used for reinforcing polymer melts. Successfully, this has been used to make a composite with PEEK and provide a better osteointegration of the implant. HAp is usually synthesized following three different routes: dry, wet, and high temperature. Dry methods use calcium and phosphate precursors that are either heated up or ground and compressed under heat to obtain a crystal. The wet methods have precursor solutions containing calcium and phosphate ions that are then precipitated through precise pH adjustment followed by aging, filtration, and drying. Often the obtained crystal undergoes a heat treatment or sintering around 1,000 °C. Other wet methods included hydrothermal (heated solution), hydrolysis. High-temperature approaches used high-temperature reaction at 500 °C and to induce the formation of HAp crystals then sintering above 1,000 °C of solution or solid salts [16–18].

Table 3.3: The different phases of calcium phosphate and their occurrence in mammalian tissues.

$\text{Ca}^{2+} \quad \begin{array}{c} \text{O} \\ \\ \text{O}^- - \text{P} - \text{OH} \\ \\ \text{O}^- \end{array}$			
Ratio	Name	Formula	Occurrence
0.5	MCPM Monobasic calcium phosphate monohydrate	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	
1.0	DCPA/monetite Dicalcium phosphate anhydrous	CaHPO_4	
1.0	DCPD/brushite Dibasic calcium phosphate dihydrate	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	
1.33	OCF Octacalcium phosphate	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	Dental calculi and urinary stones
1.5	α -TCP α -Tricalcium phosphate	$\alpha\text{-Ca}_3(\text{PO}_4)_2$	
1.5	β -TCP β -Tricalcium phosphate	$\beta\text{-Ca}_3(\text{PO}_4)_2$	Dental calculi, arthritic cartilage, soft tissue deposits
1.2	ACP Amorphous calcium phosphate	$\text{Ca}_x\text{H}_y(\text{PO}_4)_z \cdot n\text{H}_2\text{O}$ $n = 3-4.5$	Heart calcification, kidney stones
1.5	CPPD Calcium propposphate	$\text{Ca}_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$	Pseudo-gout deposits in synovium fluids
1.5–1.67	CDHA Calcium deficient hydroxyapatite	$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$ $0 < x < 2$	
1.67	HAp Hydroxyapatite	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	Enamel, dentin, bone, dental calculi
2.0	Hilgenstockite Tetracalcium phosphate	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	

3.3.2 Bioactive glass

In the search for an alternative to early metal implants that had a poor osteointegration, meaning a poor binding to the bone, Hench investigated different formulations of glass and their behavior in animal bone defects. The first bioactive glass melt was obtained with a composition of 46.1 mol%, SiO_2 , 24.4 mol%, Na_2O , 26.9 mol% CaO , and 2.6 mol% P_2O_5 . Based on this pioneering work, further studies have aimed at enlarging

the library of bioglass but only two compositions have been cleared for human use and shown better performance compared to usual metal implants. *In vitro* studies have been able to underlie the mechanism behind the integration of the bioglass in bone and other tissue. Once implanted, the glass will release some of its phosphate and calcium components which will stimulate the formation of hydroxyapatite on its surface (Figure 3.24). This inorganic biolayer formed at the surface of the implant will then allow for cells to attach, proliferate, and create a natural extracellular matrix which will create a strong physical linkage between the implanted bioglass and the surrounding tissue. This was particularly flagrant in the original work of Hench when the surgeon couldn't move the implanted bioglass, whereas the metal implant was loose and could be removed [19].

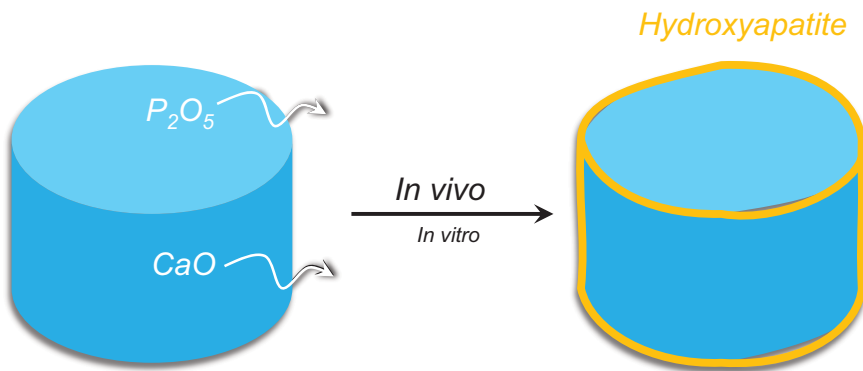


Figure 3.24: Composed of a precise ratio of silica, phosphate, calcium, and sodium, upon implantation the bioglass will release some phosphate and calcium in the surroundings which will favor the attachment of protein and the growth of the hydroxyapatite layer around it, thus favoring the osteointegration.

3.3.3 Metal

Metal has been used in biomedical applications ever since Ambroise Paré's first prosthetics in the sixteenth century. Naturally, once the surgical techniques have allowed placing implants in tissues, metal implants have emerged. For a metal implant to show a favorable response in a given biological environment in a particular function, it depends on the corrosion resistance and cytotoxicity of corrosion products. This means that the loss of metallic ions from the metal surface to the surrounding environment needs to be well understood as this can lead to degradation of the implant leading to its failure but also an allergic reaction in patients. Over the years, several alloys resisting the harsh and corrosive aqueous and salty environment of biological tissues have been developed (Table 3.4). The recent development of metal 3D printing

has opened research and development efforts in manufacturing patient-specific implants made of these biomedical metal alloys [20].

Table 3.4: Composition of the most used metal alloys for biomedical applications.

Name	Type	Composition (%)	Application
NiTiNOL	Nickel–titanium	Ni(49) Ti(51)	Shape memory stents
Ti6AL4V	Titanium	Ti(89) Al(6) V(4)	Bone implant
316L	Stainless steel	Fe(60–67) Cr(17–20) Ni(12–14) Mo (2–4) Mn(2) C(0.03)	Surgical tools, temporary implants and stents
CoCrMo	Cobalt–chromium	Co(58.9–69.5) Cr(27–30) Co(58–59) Cr(26–30) Co(45–56) Cr (19–21)	Dental, hip implant, and stents
AE21	Magnesium	Mg(97) Al(2) Rare Earth(1)	Resorbable implants

Nickel–titanium alloy (NiTiNOL)

Nitinol is an alloy made of an almost equal amount of nickel (49%) and titanium (51%). It was developed in the 1960s for military application and it was discovered that the alloy undergoes shape memory upon heat activation similarly to brass (Cu–Zn alloy) and Au–Cd alloys. But because the concentration of each metal in the alloys needs to be precisely controlled, it is challenging to manufacture. Further, due to its thermo-mechanical properties, it is also difficult to process. But for medical applications that are high added value applications, nitinol has found its main application as self-deployable stents. The stent can be folded into a small shape and introduced in the artery by a catheter and upon heat opens and pushed the artery to restore vascular flow. This technique avoids the use of inflating balloons for the deployment of the stent. The second concern for the current use of nitinol in a biomedical application is the potential allergic reaction to nickel and its carcinogenic property. To remedy to these drawbacks, nitinol stent can be oxidized to have a titanium oxide surface or can be coated with a polymer that can be further formulated with a drug to slowly elute the drug as the polymer degrades [21–24].

Cobalt–chromium alloy (CoCrMO)

Cobalt–chromium alloys possess superior mechanical properties with high corrosion resistance. They are usually supplemented with molybdenum and tungsten. These alloys have shown good biocompatibility and blood compatibility. They are used in various orthopedic, dental, and cardiovascular implants and devices and are classified into four types: ASTM F75 alloy, ASTM F799 alloy, ASTM F90 alloy, and ASTM F562 alloy with a different ratio of cobalt and chromium. The cobalt–chromium alloys are the most common alloys for dental application and hip joint replacement.

316L stainless steel

Stainless steel has been commonly used for surgical medical tools as it can be easily sterilized in an autoclave and the tools can be reused. For this application, stainless steel needs to be resistant to corrosion. The addition of 2% of molybdenum to make the 316 stainless steel improves the resistance to acids and localized corrosion caused by chloride ions. The 316L stands for low-carbon stainless steel to avoid corrosion problems caused by welding. In addition, this metal is inexpensive to manufacture and can be easily shaped by conventional forming techniques. Stainless steel implants are usually used for temporary bone fixation that is removed once the bone has healed.

Ti-based alloy (Ti–6Al–4V)

Titanium has been used for many applications due to its lightweight, resistance to corrosion, and biocompatibility. Commercially pure (CP) titaniums are classified in different grades depending on the quantity and type of impurity. The most used titanium for biomedical implants is the CP titanium grade 5 Ti–6Al–4V. However, the presence of aluminum and vanadium particles has raised some concerns as they might contribute to medical issues such as Alzheimer's and neuropathy [25].

Bioresorbable metals

Current trends in surgery aim to reduce the number of procedures and the invasiveness of these procedures. For a procedure that requires a metal implant because of the strength and load-bearing properties that are needed the development of a metal temporary implant that could be bioresorbable would avoid a second intervention to remove the supporting implants a bioresorbable. As such, magnesium, a metal naturally present in tissues has been investigated as metallic implants. But pure magnesium degrades too quickly and can release hydroxide chloride gas through reaction with water and sodium chloride present in the biological environment. To improve its corrosion resistance, alloy of magnesium-containing aluminum and rare earth have been developed. The second bioresorbable metallic materials proposed are iron implants. But the magnetic properties of irons limit its application as it is not compatible with magnetic resonance imaging techniques. Currently, these bioresorbable materials are used to make stents and screws for bone repair [26].

3.3.4 Inorganic polymers

Poly(dimethylsiloxane)

Beyond metallic and crystalline phases, polymers can also be made as inorganic materials. The most use of these inorganic polymers is Poly(dimethylsiloxane) (PDMS) which is also called silicon. It is a silicone-based polymer that is biocompatible and non-degradable. It has been used for decades for the manufacturing of microfluidic

chips and is now used in biofabrication for the fabrication of organ-on-a-chip modules. The silicone polymer is obtained through ROP catalyzed in organic solvents such as acetone by based such as potassium hydroxide and in dichloromethane with acidic sulfate and hydroxide chloride (Figure 3.25).



Figure 3.25: Ring-opening polymerization of Poly(dimethylsiloxane) using an alkaline or acidic catalyst in organic solvents.

The obtained polymer has a large molecular weight with high rotation potential through the silicone centers. It results in a flowing polymer that can penetrate thin topography, which makes it the perfect candidate for replica molding applications used to make microfluidic chips. Once the polymer is constrained within its mold, it can be crosslinked to form a strong resin. With the massive development of PDMS and variety of usage, there are many strategies for the crosslinking of PDMS. Some are water catalyzed and can be crosslinked by simple exposure to ambient air, other requires the use of metal catalyst and heat (Figure 3.26). The most frequently used crosslinkers are titanium and tin catalyst that form crosslinked resin upon heat curing and these approaches are used to make microfluidic chips, tubing, and other manufactured goods (Figure 3.27). Variation in the heat treatment time and temperature permits obtaining transparent resin of different mechanical properties and flexibility [27].

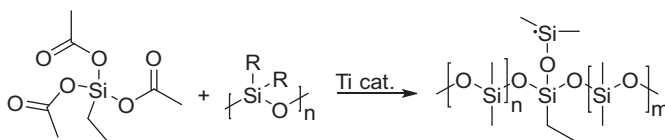


Figure 3.26: Titanium-catalyzed crosslinking of polydimethylsiloxane.

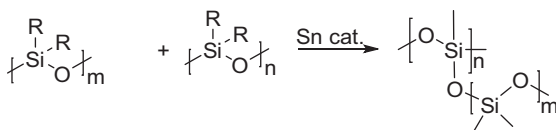


Figure 3.27: Tin-catalyzed heat curing of polydimethylsiloxane resin.

The obtained resin is an inert material that can be implanted in tissue with a limited inflammatory response. The PDMS can be processed into a different shape with micro- and nanotopography that can be further used to modulate the immune response of the implanted material. Once the resin is obtained, it can be further functionalized using

silane chemistry. Silane functionalization is obtained by oxidizing the PDMS surface with sodium hydroxide or through an oxygen plasma treatment. Then, silane can react on the freshly oxidized PDMS surface. However, as we have discussed earlier, the PDMS polymer chain is long and highly flexible. This means that the chains that are oxidized on the surface will then migrate within the bulk and the activation will not be stable. There is now a broad variety of silane that can be used for the functionalization of PDMS. The most used one is 3-aminopropyltriethoxysilane (Figure 3.28). Silanes with click chemistry moieties, epoxy, acrylate, or thiol functional groups are now available and this list is growing every year. It is thus now possible to create microfluidic chips with highly precise chemical definition and functionalization. The main application of PDMS in biofabrication is for the manufacturing of organs on-chip. These are PDMS microfluidic chips that are populated with cells or organoids that can be designed to reproduce some physiological environment for drug testing applications.

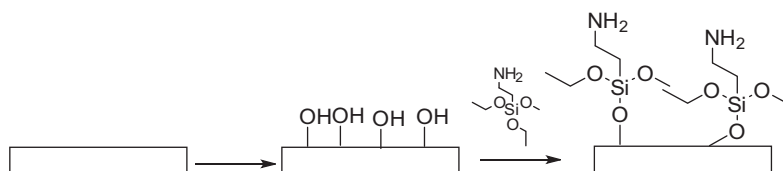


Figure 3.28: Functionalization of a PDMS resin by first oxidizing the surface with sodium hydroxide or plasma activation followed by the reaction of the hydroxyl groups with silane (here 3-aminopropyltriethoxysilane).

Polyphosphazenes

Polyphosphazenes (PPP) is an inorganic polymer that was developed in the 1960s. This is a fully inorganic polymer that degrades into amine and phosphate which are two build blocks of biochemistry. The glass transition of these polymers can be tuned by selecting different side-chain substituents and can vary between $-105\text{ }^{\circ}\text{C}$ and $30\text{ }^{\circ}\text{C}$. The PPP can be synthesized by ROP which is induced either thermally at $250\text{ }^{\circ}\text{C}$ or lower temperature (about $200\text{ }^{\circ}\text{C}$) by using a catalytic amount of Lewis acid such as aluminum chloride. The polymerization can also be conducted at room temperature using trichlorophosphoranimine and phosphate pentachloride through a mechanism of living cationic polymerization (Figure 3.29). The living polymerization approach enables, like with organic polymers, to achieve advanced polymer architectures such as block copolymer and graft polymers with comb or star architecture. The polymer is of particular interest for biomedical applications because it degrades into a building block of biochemistry amine and phosphate. The degradation rate of the polymer can be tuned by selecting different side groups. Likewise, the mechanical properties of the polymer will be determined by its organic sidechain. For instance, a thermoplastic material can be made with trifluoromethoxy side chains, a hydrogel-forming polymer can be obtained with an ethyleneoxy side chain that can be crosslinked with gamma

radiation. Their degradation into non-toxic molecules made them particularly interesting materials for drug delivery application and bioresorbable implants with a degradation rate that can occur between weeks and months depending on the side chain [28–30].

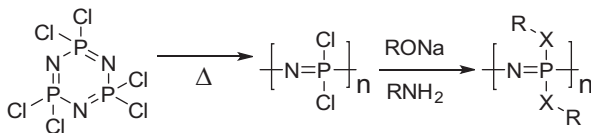


Figure 3.29: With X = O or N, the reaction scheme of the basic thermal activated polymerization reaction of polyphosphazene.

3.4 Quiz



1. What is the main mechanism for ROP?
2. Which polymer can be obtained by ROP?
3. What are the two main biocompatible inorganic polymers?
4. Why bioglass can have a better tissue integration compared to metal implants?
5. To have a bulk erosion, should the water diffusion be greater than the degradation rate of the polymer or vice versa?
6. What is the Ca/P of hydroxyapatite?
7. Which of these polymers is more hydrophobic poly(lactide acid) or poly(glycolide acid)?
8. Which one of the following polymers are made by bacteria: poly(lactide acid), PDMS, or poly(3-hydroxybutyrate)?
9. Which one of these alloys can be used for making thermally actuated metallic implants: cobalt–chromium, nickel–titanium, or titanium–aluminum–zirconium?
10. What is the definition of biomaterials?

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4 Hydrogels for biofabrication

4.1 What are hydrogels?

There are different definitions for hydrogels either based on their chemical properties or their physical properties. One definition that is widely accepted defines hydrogels as water-soluble polymers that form a tridimensional network of chains connected at crosslinking points. Therefore, if we can control the crosslinking points, we can control the hydrogels. In the following sections, we will discuss how we can create such a tridimensional network. Hydrogels have several fields of applications including food for the making of slurry or as a thickening agent, for agricultural application for the delivery of water and nutrient to the plants by reducing the evaporation rate, in biomedical applications for DNA or protein separation by electrophoresis and for wound dressing or ointment to provide hydration. In the case of biofabrication, hydrogels are highly sought after as they reproduce the natural mechanical environment of natural tissues. Overall, the worldwide market for polymer gel represents several billions USD yearly and covers many fields of application. In cosmetics, hydrogels are used to make hydrating cream. In crude oil extraction, hydrogels due to their swelling properties are used to remove water and bind hydrophilic particles. Since crosslinking points are key to controlling the hydrogel properties, we classify them by their type of crosslinking: chemical or physical (Figure 4.1).

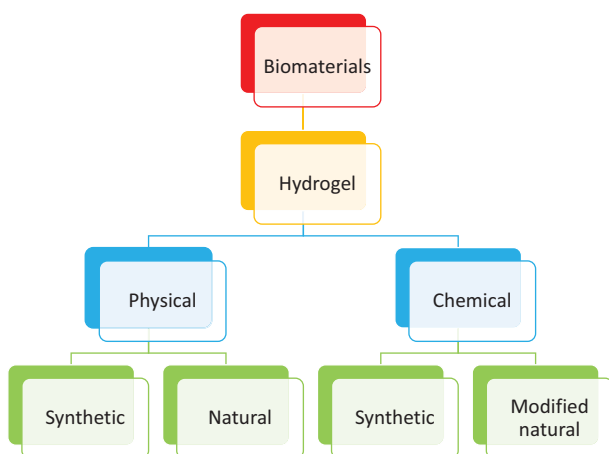


Figure 4.1: Classification of hydrogels by their mode of gelation and origin of the polymers.

4.2 Chemical hydrogels

Chemical hydrogels are formed by chemical crosslinking, meaning the crosslinking points are made through covalent bonds. These hydrogels are considered irreversible, meaning the crosslinking points cannot be removed and reformed. To form a hydrogel, we need to form a tridimensional network between the polymer chains. To do so, the polymerization needs to incorporate monomers with a functionality higher than two. Using a monomer with a functionality greater than two will allow two functional groups to be engaged in the chain growth and the remaining third functional group will be engaged in bindings between the polymer chains (Figure 4.2).

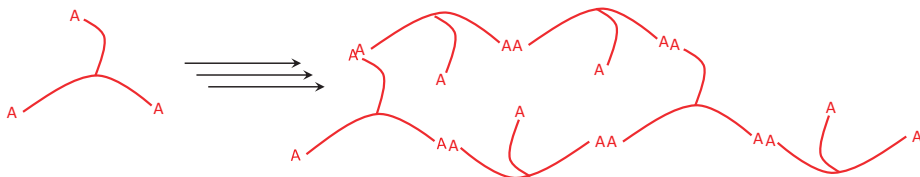


Figure 4.2: Radical polymerization approach where a monomer or monomer pool having total functionality higher than 2 can react to form a three-dimensional network.

The crosslinking of the polymer chains can be formed through two different strategies. One is to grow the polymer chain and then have a second step to create chemical bonds between the polymer chain. A second strategy consists in crosslinking the polymer chains during the chain growth.



4.A Swelling

Hydrogels composed majorly of water can swell as they can uptake water after their formation. By doing so, they will increase volume and mass using the equation below. The difference of mass can be measured, and from this measurement the swelling ratio of the hydrogel can be calculated. This is of particular importance superabsorbents such as a poly(acrylic acid) polymer that can uptake many times their weight in water (Figure 4.3).

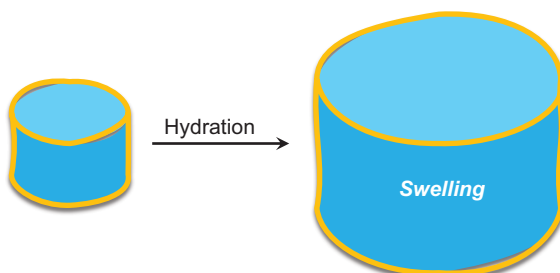


Figure 4.3: Swelling of hydrogel.

$$SW\% = \frac{(m_{\text{swollen}} - m_{\text{not swollen}})}{m_{\text{not swollen}}}$$

4.2.1 Poly(acryl amide)

Poly(acryl amide) (PAA) are hydrogels that are prepared by radical polymerization in water, following the first strategy presented above. Here, a total functionality of 3 is brought by using a combination of monomers: one monofunctional acrylamide and a bifunctional bisacrylamide (Figure 4.4). The polymerization is initiated by the addition of ammonium persulfate and the base tetramethylene diamine (TEMED). TEMED catalyzes the decomposition of the persulfate to give a free radical that will then transfer to the acrylamide and bis-acrylamide monomers. Adjusting the ratio of acrylamide and bisacrylamide allows the creation of hydrogels of different pore sizes and mechanical properties. This polymer is used for making hydrogel for electrophoresis but also to

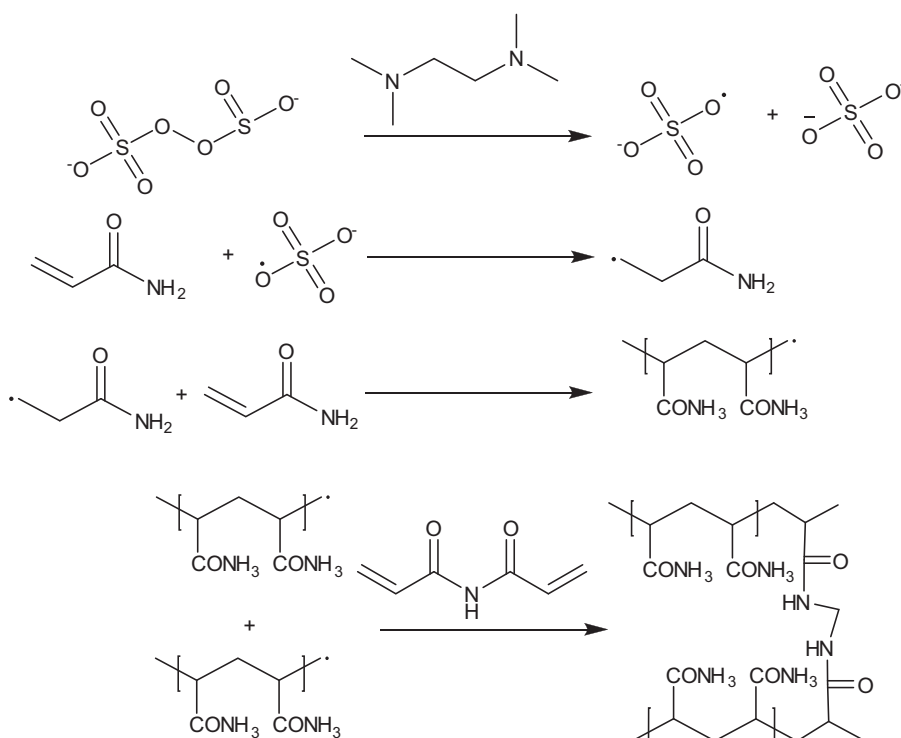


Figure 4.4: Polymerization of poly(acryl amide) three-dimensional hydrogel through radical polymerization.

make surfaces of various elastic moduli to grow cells on it. However, as for many polymers, acrylamide monomers are toxic and thus are not compatible with an in situ reaction. Meaning, all the monomers need to have reacted and need to be removed before having the hydrogel in contact with any living tissue or organism.

4.2.2 Poly(ethylene glycol)

Poly(ethylene glycol) (PEG) is a water-soluble polymer that can form a thick viscous solution at high polymer concentration. Crosslinked PEG hydrogels can be formed by reacting monomers with functionality greater than two. Using monomers that have three, four, or five branches or arms each of the arm terminated with a reactive end groups such as thiol or vinyl sulfone. For instance, mixing monomers with 4-arms that are terminated with vinyl sulfone with monomers with 4-arms that are terminated with thiol functional groups leads to a chemically crosslinked hydrogel through the reaction of thiols with vinyl sulfone (Figure 4.5). This approach can be applied with different functional groups that have different kinetic reaction rates, such as maleimide with thiol or carboxylic acid with an alcohol. Using coupling chemistry that is not toxic and that can occur in aqueous media under physiological conditions allows for the polymerization of these hydrogels in situ or with cells upon addition of the second pre-polymer (for instance (PEG-VS)), the two precursors react with each other forming an hydrogel that encapsulate the cells. Meaning, cells can be suspended into a hydrogel precursor solution that contains one of the PEG reactive pre-polymer into the three-dimensional hydrogel network.

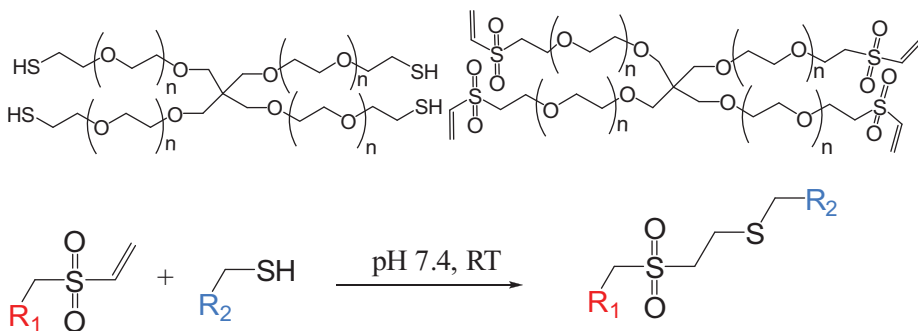


Figure 4.5: Polymerization of PEG hydrogel through a step-growth polymerization as presented in Figure 4.2.

4.2.3 Gamma irradiation

For a medical device or an implant to be used for biomedical applications, be it *in vivo* or *in vitro*, one must sterilize the objects or instrument before its use. Several methods are available including chemical (ethylene oxide) and physical (heat, ultraviolet light). The method of sterilization must be carefully chosen to make sure that the device is not impacted by the sterilization technique. For instance, using steam sterilization (121 °C for several minutes) on a thermoplastic material such as polycaprolactone, which has a T_m of 60 °C, will destruct the device. One of the techniques of choice for sterilization of industrial equipment after their manufacturing and packaging is γ radiation. Gamma rays are generated by a radioactive source and unlike α and β radiation, γ radiation can only be stopped by thick dense materials such as lead or concrete. The γ radiation forms by the radioactive decay of atomic nuclei and it is the shortest electromagnetic wave of about 8 MeV. But for this type of sterilization to be successful, the materials be sterilized must be able to sustain irradiation. During the gamma irradiation process, radicals are formed that then can induce the polymerization or crosslink of polymer chains (Figure 4.6). Conversely, this can be used as an advantage where a hydrogel must be crosslinked for instance for the crosslinking of PVP hydrogels. In the PVP case, the final step of crosslinking and sterilization occurs in a single step after manufacturing and packaging. Sterilization facility using γ radiation can accommodate whole palettes per process. This is particularly efficient for the poly(styrene) plasticware commonly used in biology laboratories where a whole shipment can be sterilized in their packaging by gamma radiation.



Figure 4.6: Scheme of the chemical reaction occurring between two polymer chains forming radical by γ radiation.

Poly(vinyl pyrrolidone)

Poly(vinyl pyrrolidone) (PVP) is usually crosslinked by gamma radiation. It can then be processed as a firm hydrogel at the same time as undergoing sterilization. Its main application is for binders in pharmaceutical applications. The most commercially successful use of PVP is for its formulation with iodine to form the disinfectant betadine. To form hydrogel PVP has been often used as a blend with poly(vinyl alcohol) (PVA) or polysaccharides such as chitosan and cellulose. It is now used in many wound dressing formulations for its ease of production due to the final sterilization that is combined with the crosslinking process.

Poly(acrylic acid)

Poly(acrylic acid) (PAA) is a polyelectrolyte type of polymer which makes it particularly suitable for holding water. It is the main polymer used for superabsorbent applications in diapers and hygienic products. It is not biodegradable and is polymerized by free radical polymerization with initiators such as azobisisobutyronitrile or potassium persulfate. The resulting polymer is biocompatible, water soluble, and has a glass transition around 100 °C. PAA can form hydrogel by absorbing water, but it often requires to be crosslinked to reinforce the polymer network. This can be achieved by gamma irradiation of the polymer in solution as presented above [8].

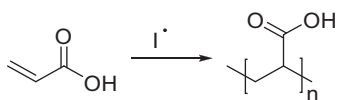


Figure 4.7: Scheme of the free radical polymerization chemical reaction to make poly(acrylic acid).

Further formulations of the acrylic polymers can be made with similar monomers including methacrylic acid and 2-hydroxyethyl methacrylate (HEMA). During the polymerization, the chains can undergo crosslinking instead of doing cyclization and thus form a three-dimensional network. Furthermore, HEMA can undergo photocrosslinking when used in conjunction with a photoinitiator and thus has found application in 3D printing.

4.2.4 Protein crosslinking

Not only synthetic polymers can be chemically and irreversibly crosslinked. Natural polymers can also be irreversibly crosslinked. Proteins are a perfect example. Made of amino acids with side chains made of reactive functional groups, these can be reacted with molecules with a functionality of two to crosslink the polymer chains. The main reactive functional groups in proteins are amine, which reacts rapidly with aldehyde and acetal.

Glutaraldehyde

Composed of an alkane chain terminated by two aldehyde functional groups, each molecule of glutaraldehyde can react with two polymer chains by a reaction with an amine (Figure 4.8). However, glutaraldehyde is cytotoxic and thus cannot be used for the crosslinking of hydrogel-containing cells, nor can it be used without proper removal of the residual unreacted glutaraldehyde. It is usually used to crosslink gelatin or collagen-based hydrogel or sponges. For the crosslinking of gelatin sponges, the gelatin hydrogel is dehydrated by freeze-drying and then crosslinked with glutaraldehyde. But

the main usages of glutaraldehyde remain for the fixation of biological samples for staining in histology.

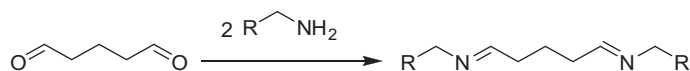


Figure 4.8: Scheme of the chemical reaction of glutaraldehyde with two molecules containing primary amine.

Genipin

Found in the fruit extract of *Genipa americana*, also called *Jagua azul* in Mexico, the genipin molecule can react with two primary amines. As such, it can be used to crosslink proteins by reacting with the amine side chains. It is much less toxic than glutaraldehyde and thus can be used for biomedical applications with less stringent precautions (Figure 4.9).

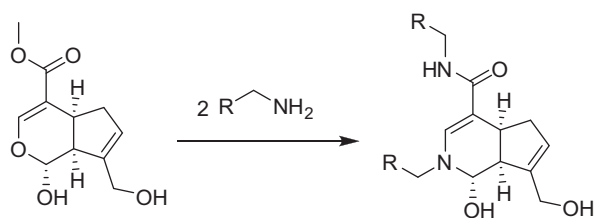


Figure 4.9: A. Scheme of the chemical reaction of the crosslinking of two protein molecules using genipin, a naturally occurring compound.

4.2.5 Photocrosslinking

Another method to induce the formation of radicals is the use of a photoinitiator derived from free radical polymerization. To date, there are several photoinitiators available that upon illumination with the correct wavelength decompose into two radical molecules. The radical once created can then propagate to the polymer or reactive species to form radical that can polymerize and crosslink into a hydrogel. One of the most well-known photopolymers used in biofabrication is gelatine methacrylate (Figure 4.10) (GelMA). It is a gelatine hydrogel that has been modified with methacrylate functional groups. This can be achieved by reacting the gelatine polymer that has an amine side group from the amino acid with anhydrous methacrylate. Beyond gelatine, other natural polymers have been methacrylated using the same process with anhydrous methacrylate: hyaluronic acid, alginate, chitosan, and collagen.

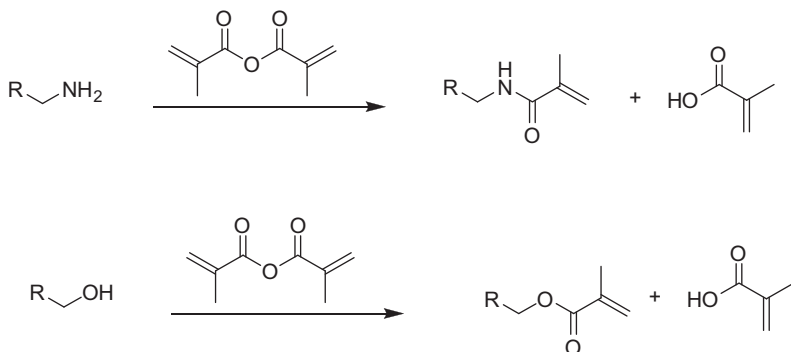


Figure 4.10: Scheme of the chemical reaction of methacrylate of an amine and alcohol functionalized polymer.

A photoinitiator is needed to create a radical. The most used photoinitiators are Irgacure 2959 (LAP) which is activated by light at 365 nm, and lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP) at 405 nm (Figure 4.11).

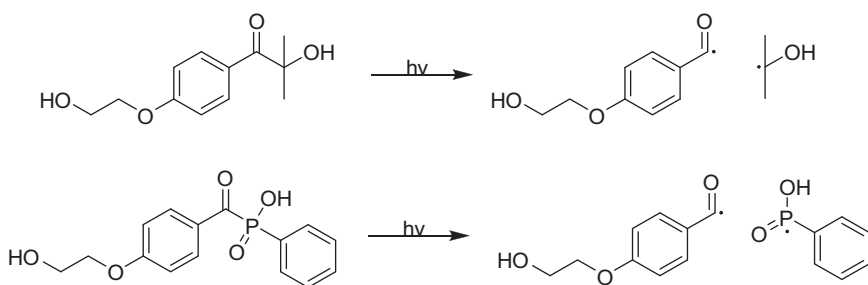


Figure 4.11: Mechanism of a radical formation of a photoinitiator Irgacure (up) and LAP (down).

Once activated, the photoinitiator propagates the radical to methacrylate groups which will, in turn, react with each other thus crosslinking the polymer chains (Figure 4.12). These sorts of systems are used for bioprinting. GelMA can be extruded in a bioprinter and then crosslinked post-extrusion to fix the printed hydrogel. In stereolithography bioprinting, a similar system can be used. However, here, one

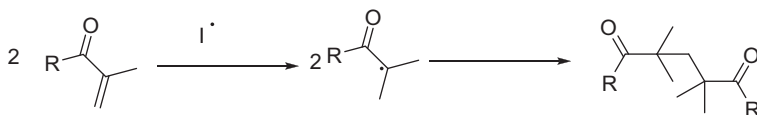


Figure 4.12: Crosslinking of a methacrylate polymer with a photoinitiator.

must be aware of the propagation of the radical beyond the illumination volume, called “shadow cure,” and this can lead to a loss of resolution. These bioprinting processes will be discussed in more detail in the following chapters.

Further development of the photocrosslinking of hydrogel leads to the implementation of more complex systems. Great efforts have been focused on creating photoinitiator systems excited by visible light. Among them, Eosin-Y, riboflavin, and camphorquinone are good candidates to substitute the conventional UV-activated systems. Indeed, UV irradiation is possibly harmful to the encapsulated cells. However, in comparison to UV light photoinitiators, visible light photoinitiators are rapidly cured under normal lighting conditions, as the energy level of the visible light is lower than that of UV light [1]. Another system, the two-photon polymerization (2PP) has been promising for the photocrosslinking of hydrogels to increase the printing resolution. 2PP utilizes high power near-infrared or infrared femtosecond lasers with initiators that generate radicals after absorbing two photons, unlike UV-vis-based photopolymerization where initiators produce radicals after one-photon absorption. If sufficient light power is provided at the focal point, a free radical can be initiated. This means that in 2PP, excitation only occurs where the laser beam is sharp which allows the precise spatial crosslinking. 2PP uses a laser between 700 and 1,050 nm in combination with initiators that are highly conjugated such as rose Bengal, benzophenone, or flavin adenine [2].

4.2.6 Click chemistry

Alternative crosslinking methods that rely on click chemistry reactive functional groups have emerged in the past decades. Click chemistry was defined by Sharpless in 2001 as reactions that are high yielding, stereospecific, simple to perform in a benign solvent, and create only by-products that can be removed without chromatography. Further development of these chemistries has led to the creation of a wide library that is constantly growing as summarized in Table 4.1. Click chemistry reactions are attractive for the crosslinking of hydrogels because the reactions can take place in water and do not have by-products, offering the possibility to create a system where two pre-polymers are mixed to form a hydrogel. These moieties have been extensively used for the functionalization of branched PEG. Each of these systems has a different reaction kinetic. Also, because these moieties are extremely reactive, some of these functional groups can have limited stability under physiological conditions. For instance, the system maleimide–thiol (Michael addition) reacts in seconds, but the maleimide group is stable only a few minutes at pH 7.4. Other systems like the inverse electronic demand Diels–Alder are reversible and have been used to make self-healing hydrogels. The hydrogel cut in two can be reformed into one piece of the hydrogel by rearrangement of the polymer chains and reaction of the diene with the dienophile [23]. Some of the cycloaddition moieties such as the strand

Table 4.1: Click chemistries systems for the crosslinking of polymers to form hydrogels.

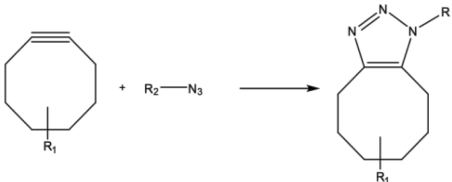
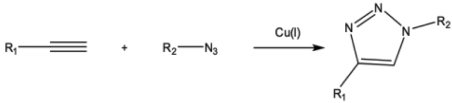
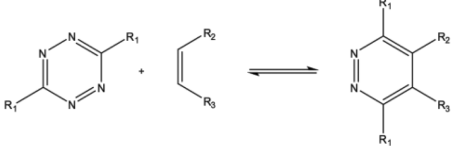
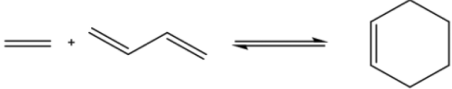
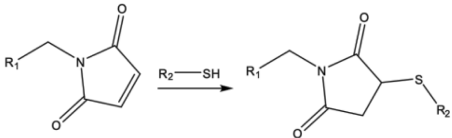
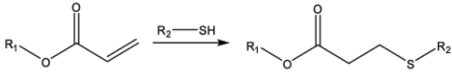
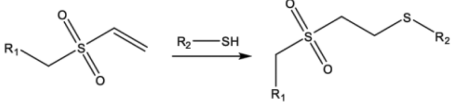
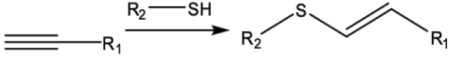
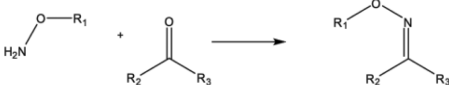
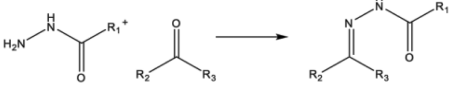
Name		Reaction mechanism
Cycloaddition	SPAAC	
	CuAAC	
	IEDDA	
	Diels–Alder	
Thiol reactive	Michael addition	Thiol–maleimide
		
		Thiol–acrylate
		
		Thiol–vinyl sulfone
		
		Thiol–yne
		

Table 4.1 (continued)

	Name	Reaction mechanism
Aldehyde	Oxyme	 $\text{H}_2\text{N}-\text{O}-\text{R}_1 + \text{R}_2-\text{C}(=\text{O})-\text{R}_3 \longrightarrow \text{R}_1-\text{O}-\text{N}=\text{C}(\text{R}_2)-\text{R}_3$
	Hydrazone	 $\text{H}_2\text{N}-\text{N}(\text{H})-\text{C}(=\text{O})-\text{R}_1 + \text{R}_2-\text{C}(=\text{O})-\text{R}_3 \longrightarrow \text{R}_2-\text{C}(\text{N}=\text{N}-\text{C}(=\text{O})-\text{R}_1)=\text{C}-\text{R}_3$

promoted are highly reactive but require multistep reaction to obtain the final product and, thus are now expensive and limited for highly valuable applications that needs only a limited amount of reagent such as for microscopy labeling experiments. Further development of the click chemistries systems and new synthesis routes for these now expensive moities will lead to reducing cost and the opportunity to use these for hydrogel formation. Another use of these chemistries is for the functionalization of hydrogel with biological molecules. For instance, azide functionalized proteins can be reacted with alkyne functionalized hydrogel with a copper catalyst to form biologically defined hydrogels.

4.3 Physical hydrogels

Synthetic polymers can form reversible hydrogels through non-covalent crosslinking or non-reversible hydrogels upon chemical crosslinking of the polymer network. Other polymers such as PVA form physical hydrogels through hydrophobic interactions, and has been used for the generation of 3D cartilage microenvironments [5]. A distinct advantage of synthetic polymers over naturally sourced biopolymers is the relative ease with which the polymers can be engineered for a dedicated application by modifying their chemical structure, and in turn, physical properties. Likewise, natural polymers can be crosslinked chemically as described earlier in this. But most of the natural polymer forming hydrogel occur through physical crosslinking.

4.3.1 Synthetic physical hydrogels

Poloxamer

These are a family of block copolymers formed from poly(ethylene oxide) and poly(propylene oxide) (PPO) marketed under the name of Pluronic. To date, there

exists a great variety of these polymers as many combinations of and PPO can be made. Indeed, the molecular weight of each block can be varied to modulate the gelling properties of the polymer. Their main use is in food and texture modifiers for the cosmetic industry but they also seen great interest for biomedical applications.

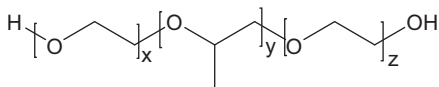


Figure 4.13: Pluronic block copolymer chemical structure made of poly(ethylene oxide) and poly(propylene oxide).

So, the PPO-PEO combination called F127 gives a block copolymer with a lower critical solution temperature (LCST) meaning that at low temperature the polymer is in solution and at high temperature, a gel is formed. Depending on the concentration of the polymer in the solution other organizations of the polymer chains can be obtained including micelles. This organization is due to the aggregation of the PPO chains that are more hydrophobic than the PEO chains (Figure 4.14). An interchain hydrophobic organization that is driven by the temperature and the concentration of the polymer in solution leads to the micellar solution or gel organization of the polymer. Typical hydrogel formation of Pluronic F127 is obtained for solutions concentration above 30% w/w at a temperature above 20 °C, transition from gel to solution occurs at 4 °C or by boiling off the gel.

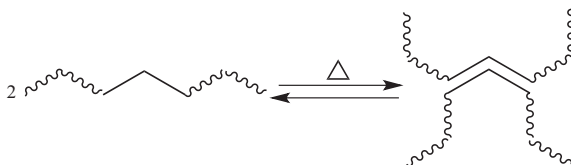


Figure 4.14: Pluronic undergo a thermally-controlled gelation by the aggregation of the hydrophobic block into a crosslinking point when the polymer is solubilized in water.

4.3.2 Natural physical hydrogels

Natural polymers that have been used as synthetic ECM scaffolds are hydrogel-forming biomaterials originating from animals, plants, or microorganisms. These polymers are either polysaccharides or proteins and some of them can be efficiently sourced and obtained in high purity. However, because of their origin, one must be aware of the potential batch-to-batch reproducibility which can be dependent on the source of raw materials or external factors. For instance, algae-extracted polysaccharides can have seasonal variability and their properties can depend on their harvesting area.

4.3.3 Polysaccharide-based (vegetal)

Cellulose

Cellulose is a naturally occurring polysaccharide composed of a repeat unit of β -D-anhydroglucose and can be obtained from plants, algae, and bacteria (Figure 4.15). Hydrogels are made from nanocellulose colloids, which are solid particles dispersed in water. Nanocellulose is obtained through the chemical depolymerization of the raw polysaccharide. The obtained colloids behave like a fluid in the diluted state and like a gel at higher concentrations. The obtention of a hydrogel from the colloidal dispersion of nanocellulose is mainly dependent upon the aspect ratio and volume fraction of the colloids. The colloidal dispersion confers these hydrogel shear-thinning properties. Such properties enable the hydrogel to be injected through small needles and this is a highly attractive property as a rheology modifier in 3D printing inks. However, in contrast to other polysaccharide gels, such as agarose, cellulose hydrogels are not highly stable and can be redispersed in water by shearing. Therefore, the formulation of pure nanocellulose hydrogel is limited and it is often combined with another polymer-forming hydrogel.

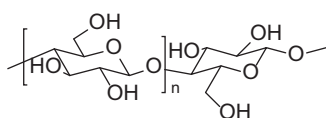


Figure 4.15: Chemical structure of cellulose.

Alginate

One of the most used polysaccharides in biofabrication is alginate, which gels upon complexation with divalent cations. The polysaccharide structure varies greatly depending on the seaweed growth environment, algae species, and extracted algae tissue. It is composed of two saccharides, the mannuronate (M) and guluronate (G) arranged in sequences of M- and G-block regions and randomly inserted M and G units (MG-blocks) (Figure 4.16). The M/G ratio is defined by the ratio of mannuronate to guluronate.

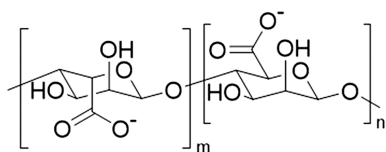


Figure 4.16: Chemical structure of alginate.

Alginate forms a gel in presence of divalent cations such as Ca^{2+} , following the egg-box model. The divalent cations complexes with the carboxylate groups of the G-blocks, while the M-blocks have a lower affinity to complexation (Figure 4.17). Because cations have a higher affinity of divalent cation toward the G-blocks, the gel properties will

greatly depend on the M/G ratio and the G-blocks length. Alginate with higher G content and a low M/G ratio will therefore produce a stiffer gel with higher gel strength than alginate with a high M/G ratio. Therefore, it is critical to know the M/G ratio to have reproducible rheological properties of the formed hydrogel. Finally, the nature of the cation will also affect the hydrogel strength. For instance, Ca^{2+} exhibits stronger interactions with the alginate than Mg^{2+} .

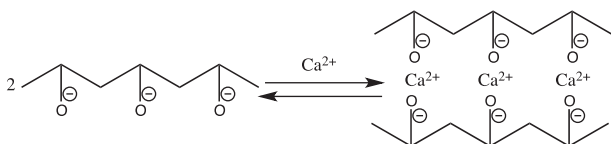


Figure 4.17: Alginate crosslinking mechanism through the complexation of divalent cations, here Ca^{2+} .

Agarose

Extracted from red seaweed, agarose is isolated from agar, a mixture of polysaccharides including agarpectin, a non-gelling polymer. Agarose's backbone is composed of β -D-galactose and 3,6-anhydro- α -L-galactose (3,6 AG) (Figure 4.18). Changes in the composition and structure of agarose polysaccharides such as the presence of α -L-galactose and other minor substituents (sulfate, methyl ether, pyruvic acid) [9] are known to occur depending on the species [9, 10] and seasons [11, 12].

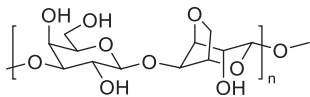


Figure 4.18: Chemical structure of agarose.

The composition of agarose controls the formation of secondary structures of the polysaccharide which then impacts its gelation mechanism that occurs through a phase separation mechanism, involving the formation of double helices in the polymer backbone and aggregations of these helices into crosslinking points creating a 3D hydrogel network (Figure 4.19). Agarose is a thermoreversible hydrogel that has been widely used for electrophoresis. It is an interesting hydrogel for biomedical applications because it exhibits hysteresis. The transition from solution to gel occurs at a lower temperature (below 37 °C) than the transition from gel to a solution (above 85 °C). It is therefore stable at physiological temperature and forms a translucent hydrogel. There are to date several extraction methods for agarose and each type of extraction can lead to a polysaccharide with different gelling temperatures. Agarose can be obtained as a low melting and high melting temperature hydrogel. Due to its availability and specific mechanical properties, agarose has been a material of choice

for the generation of 3D cell microenvironments for the culture and differentiation of chondrocytes [13].

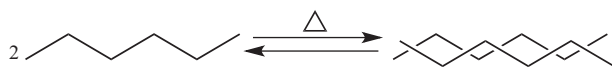


Figure 4.19: Agarose crosslinking mechanism through the aggregation of secondary structure that can be disrupted by heat.

κ-carrageenan

Carrageenans are sulfated polysaccharides extracted from red seaweed. Their backbone is composed of β-D-galactose and 3,6-AG with sulfate groups on the galactose units [14] Figure 4.20. They form hydrogel through an alpha-helical aggregation, like agarose. The resulting hydrogel is strong and brittle. Like other algae-extracted polysaccharides, factors such as species seasons growth conditions, and extraction conditions are influencing the 3,6-AG and sulfate content which in turn alters the helix formation leading to different gel properties [21].

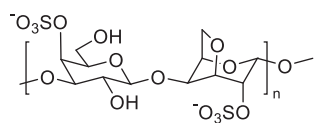


Figure 4.20: Chemical structure of the repeat unit of κ-carrageenan.

Unlike agarose, κ-carrageenan goes through a coil-to-helix transition upon addition of cations, leading to the formation of double helices. In κ-carrageenan, the helix formation is followed by further helix aggregation [22]. The type of cations used to induce the gel formation will impact the mechanical properties of the hydrogel. For instance, κ-carrageenan forms a stronger gel with K^+ than with Na^+ [25]. Not only cations but also some anions such as I^- and SCN^- have been reported to bind to the helix influencing the gelation mechanism by impeding helix aggregations and gelation.

4.3.4 Polysaccharide from microorganism

Dextran

Beyond plants and algae, microorganisms also produce polysaccharides that can form hydrogels, and these can be used for biomedical applications. Dextran is obtained from lactic acid bacteria such as *Leuconostoc mesenteroides* or mutants *Streptococcus sp.*, and is widely used in medicine since the early 1960s as a blood volume expander. Low molecular weight Dextran is also used to increase blood flow and reduce blood viscosity. Dextran neutral polysaccharide chain is composed of α-glucan with glucopyranoside side chains (Figure 4.21). It is freely soluble in water but can be formulated as

a hydrogel at a higher concentration. With the use of dextran for medical applications came the establishment of a supply chain of medical-grade materials. Naturally, dextran has fuelled some hydrogel-based innovation for drug delivery systems or cell encapsulation. But being synthesized by bacteria with many side chains, in comparison to linear polymers, it is often difficult to have a precise characterization of the polysaccharide and all its side chains. This made it rather challenging to chemically modified for further applications.

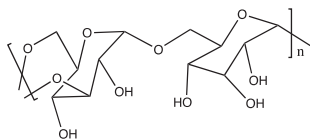


Figure 4.21: Chemical structure of the main linear chain of Dextran.

Gellan gum

The negatively charged polysaccharide gellan gum (GG) is produced by *Sphingomonas elodea* found in the lily plant. Initially used as a replacement for agar for microbiological culture, it is mainly used as a food thickener. The backbone repeat unit is composed of glucose, glucuronic acid, and rhamnose which allow for the easy functionalization of the backbone through reaction with the carboxylic acid of the glucuronic acid (Figure 4.22). The hydrogel formation is thermoreversible and the gel is believed to aggregate through the formation of a double helix and subsequent coil aggregation. The addition of cation to the solution reinforces the hydrogel which is necessary to reach a high gel strength. It has seen research application in the field of tissue engineering for cartilage repair and bone by incorporating inorganic phases such as hydroxyapatite [28]. Due to its use as a food additive, research has been conducted to use it in the formulation of drug delivery systems. Because it can be produced in high quantity in a reproducible manner, GG could be a good candidate for biofabrication application.

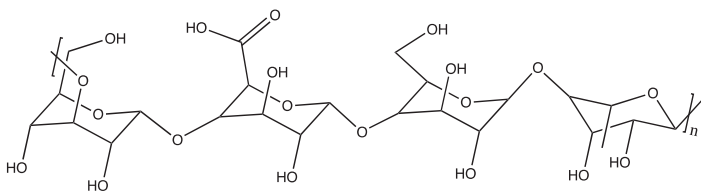


Figure 4.22: Chemical structure of Gellan Gum.

Xanthan gum

A widely used food stabilizer and formulation thickening extracted from the bacteria *Xanthomonas campestris* is xanthan gum (XG). The repeat unit is quite complex

and is composed of a glucose backbone, mannose, and glucuronic acid side chain attached to every other glucose [29]. The organization of the backbone chain impacts the gel-to-sol transition temperature. It is a versatile polysaccharide for biomedical applications [30]. The hydrogel formation can be triggered by divalent cation through the complexation with the glucuronic acid sugar. Alternatively, crosslinking is possible through inorganic sodium triphosphate or after chemical modification of the backbone by an organic crosslinker such as methacrylation. The XG formulated with hydroxyapatite phase was proposed for a bone implant and blends of XG with other polysaccharides were investigated for tissue engineering applications *in vivo* [31]. Due to its low cytotoxicity and low immune response, XG is a material to be taken into consideration for biofabrication.

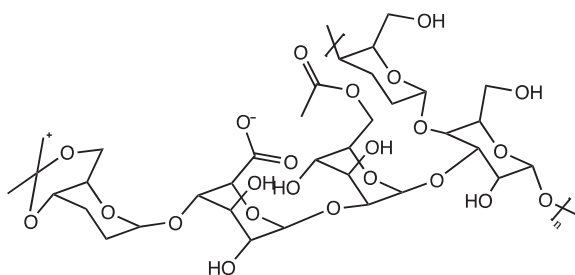


Figure 4.23: Chemical structure of Xanthan polysaccharide.

4.3.5 Polysaccharide from animal

Chitosan

An example of a polysaccharide sourced from animals is chitin, which is extracted in its deacetylated form as chitosan, a material forming crustacean shells, a by-product of the food industry [32] (Figure 4.24). Chitosan-based hydrogels are stable under physiological conditions and have been shown to possess antimicrobial properties, as well as blood clotting properties [33]. It is now possible to obtain chitosan as a medical-grade material produced according to good manufacturing procedures. Thus, it is to be expected that new products incorporating chitosan in their formulation will come to the market. It is the only polysaccharide in this list being positively charged and having an amine on its repeat unit. This is of particular importance as it allows for the easy functionalization of the polysaccharide through alternative synthetic routes as with the other polysaccharides listed above. Chitosan hydrogel can be formed by various routes either physical through the blending with glycerophosphate and chemical crosslinking by glutaraldehyde reaction with the amine groups for instance.

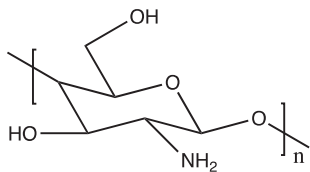


Figure 4.24: Chemical structure of chitosan repeat unit.

4.3.6 Protein-based hydrogels

Fibroin, Silk

The most common producer of fibroin is the *Bombyx mori* worm which produces the silk used in textile (Figure 4.25). It produced the protein fibroin that can be processed into silk fibers. The organization of the protein into anti-parallel β -sheets makes it a resistant fiber that was used for the manufacturing of textile. Once extracted, the protein can be processed into a hydrogel [35]. Fibroin is known to be safe, non-cytotoxic and many companies are now scaling up the production of medical-grade materials. In its regenerated solution, fibroin will form hydrogel at physiological temperature. However, the physical hydrogel is regarded as a weak hydrogel for many applications in tissue engineering. Indeed, the resistant fibers need to be dissociated to be solubilized and this can alter its structure. It is therefore often further crosslinked using chemical methods. Alternatively, the hydrogel can be processed as a carrier for drug delivery application, as a thickening agent, or be processed as a porous sponge.

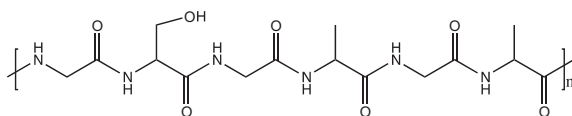


Figure 4.25: Chemical structure of the repeat unit of fibroin protein.

Gelatin

Gelatin is a transparent colorless protein derived from collagen extracted from bone and cartilage of animal tissues. It is obtained by chemical or physical treatment (heat) of bones, which partially hydrolyze collagen present in the tissue. The protein obtained is not able to reform the full triple-helix distinctive of collagen proteins. Gelatine forms a gel at room temperature and is used in many cosmetic formulations, food additives, and more recently as a material of choice for biofabrication. However, the hydrogel formed by gelatine is quite weak. Therefore, gelatine is mostly used in its chemically modified methacrylate form (see paragraph above) which permits chemically crosslinking of the hydrogel by photopolymerization of the methacrylate functional groups. Although its application for medical application raises some

concern in terms of the safety profile of the animal extracted materials, it has found a niche application in the bioprinting of tissue models for *in vitro* application. These model tissues are not intended for implantation and thus GelMA is adequate.

4.B Viscosity

It is the measure of the resistance of fluid or a solution of polymer to deformation. It is the physical expression of the thickness of a solution. For instance, mixing water and plain flour would give a thick solution as the ratio of flour increases compared to water, the solution becomes thicker and thicker until it flows extremely slowly that it is not visible to the naked eye. Similarly, one can say that ketchup is thicker than water. We would say that the viscosity of water is lower than this of ketchup. The viscosity will correlate with the capacity of a solution or blend of the solution to flow. If we apply a force to a viscous material, this material will move in the direction of the force (Figure 4.26).

i

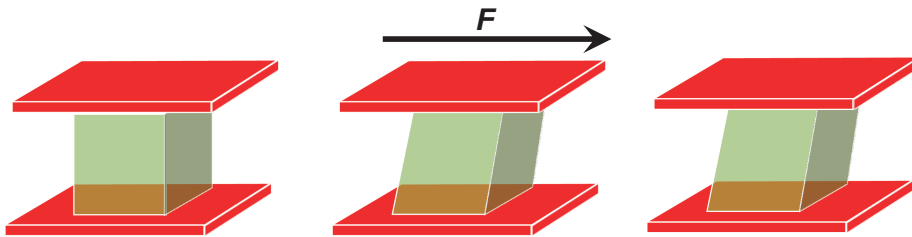


Figure 4.26: Hydrogel viscosity. The force applied to the material irreversibly deforms the hydrogel.

4.C Elasticity

The elasticity is the ability of a deformable body to resist the distorting effect of an applied force. An elastic material that has an elastic behavior will regain its original force upon the application of a force. If we take a hydrogel and apply a shearing force to start its flowing, if the hydrogel has an elastic behavior it will not move, but deform and regain its original shape (Figure 4.27).

i

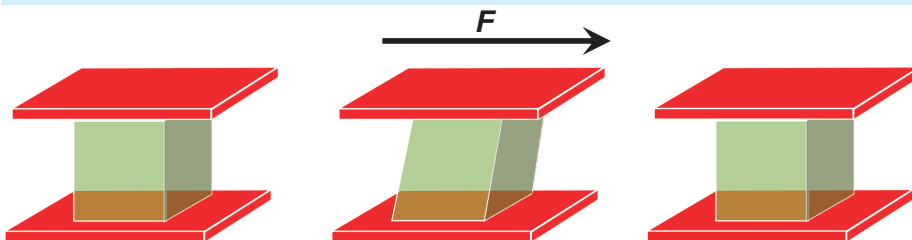


Figure 4.27: Hydrogel elasticity. The force applied to the material deforms the hydrogel that comes back to its initial shape.



4.D Viscoelastic behavior

Hydrogel, being a solid that has high content of water, has some of the properties of water and some of a solid. Meaning that they have a viscous behavior (from the water) and an elastic behavior (from a solid). As such, we say that they have a viscoelastic behavior or that they are viscoelastic solids. But to better describe these materials, one wants to know if they are more viscous than elastic. These means are they flowing or are they staying as a solid and do not flow. To define this, the complex shear modulus is used (Figure 4.28). The complex shear modulus describes the elastic (G') and viscous (G'') components of the material into a vector. The phase shift reflects the ratio of a viscous component with an elastic component.

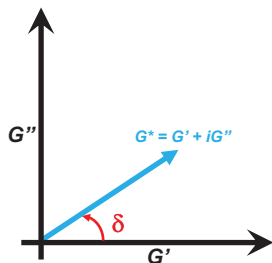


Figure 4.28: Mathematical representation of the shear (G') and loss (G'') modulus.

4.4 Quiz



1. What is the difference between physical and chemical hydrogels?
2. What is the minimum functionality required to polymerize a crosslinked polymer network?
3. What are the two components used to describe the mechanical behavior of hydrogels?
4. What are the three possible sources of hydrogels?
5. Which sugar unit is responsible for the chelation with cation in alginate?
6. Which functional group is used to create photocrosslinkable hydrogel from gelatine?
7. We measure the mass of a hydrogel at 34 g. In a container, we submerged this hydrogel for 2 h. We measure again the mass of the hydrogel and it has increased to 45 g. What is the swelling percentage of the hydrogel?
8. What is the LCST?
9. Give an example of a polymer having an LCST
10. Why does agarose has a hysteresis behaviors when it comes to its sol–gel and gel–sol temperature?

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5 Synthetic and natural extracellular matrix

5.1 What is the ECM?

The extracellular matrix (ECM) is defined as the non-cellular portion of tissue. It is made of an ensemble of macromolecules that are making the three-dimensional network that provides structural and biochemical support to the cells (Figure 5.1). The ECM assumes diverse functions ranging from mechanical support, cell adhesion, and intercellular communication. In the human body, the ECM composition varies to assume the diverse mechanical and biological properties of the various tissues. For instance, the ECM of the skin will be elastic whereas the ECM of bone will be mineralized to resist compression. The ECM is mainly composed of cell-secreted macromolecules such as polysaccharides and proteins that form the hydrogel materials that provides the 3D mechanical network required for the cells to form a tissue. In addition, growth factors (GFs) and chemokines secreted by the cells are diffusing through or bound to the ECM and assure intercellular communication. Cell-secreted matrix metalloproteinase (MMP) enzymes are capable to degrade the ECM.

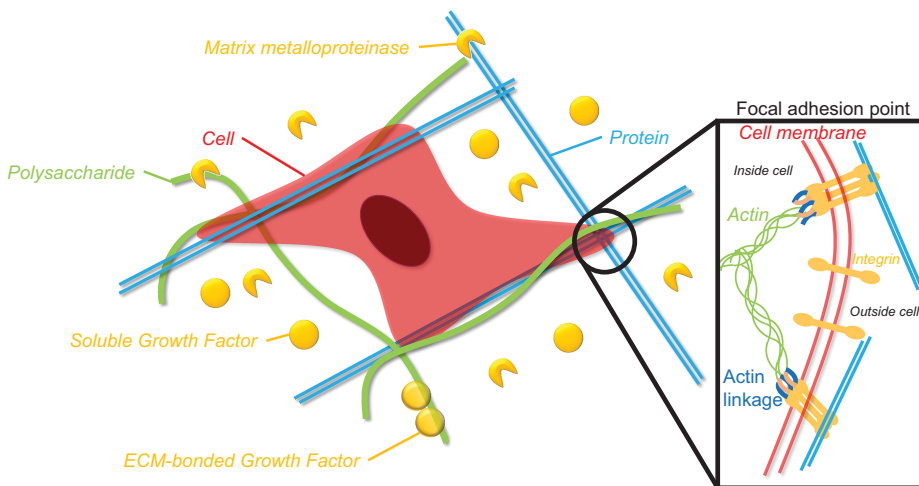


Figure 5.1: The cell in the extracellular matrix (ECM) is composed of macromolecules, growth factors, and degrading enzymes.

5.2 The composition of the ECM

The ECM is made of polymeric materials comprising polysaccharides and proteins. These biomacromolecules are secreted by cells and are assembled to form complex ECM structures with specific biological and mechanical properties (Table 5.1).

5.2.1 Glycosaminoglycans

In the ECM, glycosaminoglycans (GAGs) are anionic polysaccharides that can be subdivided into two categories: sulfated and non-sulfated. Heparin, a sulfated extracellular GAG first identified in the blood in 1919 [1], plays a major role as a blood thinner and during the wound healing process to inhibit blood coagulation. Following this discovery, a range of GAGs have been identified including hyaluronic acid (HA), which is the only non-sulfated polysaccharide found in mammalian tissues. HA is one of the major components of the ECM and plays a role in cell proliferation and migration. In particular, HA can bind to the cell receptor CD44, which is involved in cell adhesion and allows cell migration [2]. Like other GAGs found in the ECM such as chondroitin sulfate and heparin sulfate, HA exhibits specific viscoelastic properties that play a role in regulating the tissue mechanical properties such as high compressive modulus and lubrication of articulations [3]. The anionic charge of GAGs promotes binding with GFs, including the fibroblast growth factor family, allowing them to act as signaling reservoirs capable of immobilizing or releasing GFs as required [4, 5]. While already forming high molecular weight polymers, GAGs can be modified by enzymes to covalently assemble with proteins and form the proteoglycans

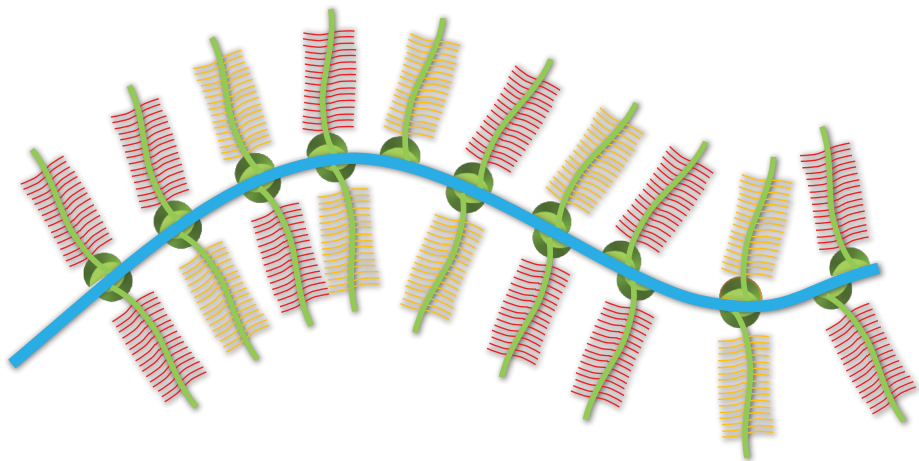


Figure 5.2: Glycosaminoglycan (GAG) formed by a hyaluronic acid backbone (blue), link to a core protein (green) on which keratan sulfate (orange) and chondroitin sulfate (red) can attach.

(PGs) super-family. In that assembly, the GAGs form branches perpendicular to a core protein and are often connected to it by a tetra-saccharide composed of a glucuronic acid (GlcA), two galactoses, and a xylose residue (Figure 5.2) [6]. However, due to the complex biosynthesis of GAGs and PGs that involve different enzymes, most of their role in the ECM has not yet been fully understood. Currently, only heparin and HA have found clinical and cosmetic applications and can be affordably sourced either as a recombinant molecule or directly from animals [7, 8].

5.2.2 Proteins

In ECM, proteins are secreted by cells and controlled by the activation of specific genes. The most abundant protein, collagen is found in different variations depending on the gene code utilized and post-transcriptional modification that occurs inside and outside of the cells. Collagen, like the protein laminin, forms a triple helix and can interact with other proteins such as nidogen which linked these two proteins together to form the basal lamina of cardiac and lung tissues [9]. The helical secondary structures of collagen and laminin produce resistant fibrillar materials that contribute to the mechanical stiffness of tissue. Similar to GAGs, proteins of the ECM can serve as signaling reservoirs by binding, and when required releasing GFs [4]. In addition, specific cell surface receptors can anchor to these proteins, including the binding of fibronectin and collagen to the integrin receptor [10–12], laminin to the 67 kDa laminin receptor (67 LR) [13], and elastin to the 67 kDa elastin receptor (67-ER) [14]. As required, cells can also secrete enzymes, MMPs, to degrade ECM proteins. MMPs are released into the ECM and upon metal complexation, cleave specific amino acid sequences in the ECM proteins. For example, collagen types I, II, and III are cleaved by MMP-1 [15, 16]. These processes provide control over the maintenance of the ECM and its degradation in a process called homeostasis.

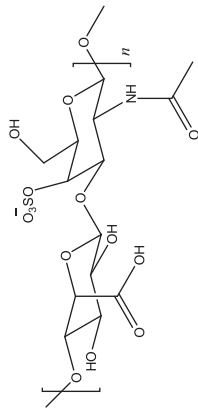
5.2.3 Proteoglycans, PGs

PGs are glycosylated proteins in which GAGs are typically covalently or physically bound to a protein core. These super-macromolecules can be very large with molecular weights over 1,000 kDa [17]. The specific biological functions of many PGs are still emerging, but interaction with GFs and cell receptors has already been identified [18]. However, the main function of PGs appears to be related to their physico-chemical properties. For example, the high surface charge of PGs allows them to retain large amounts of water through osmotic effects, making them the material of choice for hydrogel-like tissues such as articular cartilage [19].

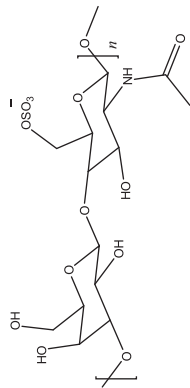
Table 5.1: Macromolecular composition of the extracellular matrix (ECM).

Name	Tissue location	Function	Structure	Mw (kDa)
Polysaccharide Heparin	Blood, wound	Anticoagulant		10–12
Heparan sulfate	Cartilage, mucosa	Compressive load Shock absorption Water retention Formation of proteoglycans Reservoir of soluble signals		10–70
Hyaluronic acid	Eye, cartilage			800
Chondroitin sulfate	Cartilage			300

Dermatan sulfate Cartilage 60



Keratan sulfate Cornea, cartilage bone 20



Protein	Stretch strength of the tissue	Protein	Stretch strength of the tissue
Collagen	Network organization	Triple strand	290
Laminin	Connect collagen and laminin	Triple strand	900
Nidogen	Protect from exterior	Single strand	150
Keratin	Elasticity of the tissue	Single strand	200
Elastin	Cell adhesion	Single strand	64*
Fibronectin		Double strand	250

*only the precursor: tropoelastin.

5.3 The tissue repartition of ECM components

To form the proper matrix that will support their function and fate, cells can synthesize these biomacromolecules in different proportions. The combination of GAGs, PGs, and proteins in different ratios allow for the formation of tissues with mechanical properties tailored to their task. For example, in articular cartilage tissue, the ability of PGs to retain large amounts of water upon compression is crucial to support the load bared by the joints [20]. In the skin, cells organize at the tissue interface to form a protective barrier in which a collagen type I matrix provides high resistance and collagen type III the flexibility [21, 22]. Similarly, the ECM of the vasculature is composed of resistant protein-based biomacromolecules, including collagen type IV and laminin. Connective tissues are hydrogel-like materials made up of fibrillar and hydrogel components that usually bind different tissues type-together [23]. Connective tissues can be easily degraded and remodeled by cells through the secretion of enzymes, such as MMPs [24].

Rearrangement of the ECM is a critical feature for the repair of injured tissue, however, the imbalance between its degradation and synthesis rates may be related to various diseases [25]. As an example, heart diseases such as myocardial infarction have been related with enhanced levels of tissue inhibitor of metalloproteinases – 1 thereby inhibiting collagen degradation leading to the formation of non-elastic tissue which deforms upon heart contraction [26].

By varying the composition of the ECM, different tissue-mechanical properties can be achieved. From the smoothness of soft brain tissues to the toughness of bones, the mechanical properties of tissues vary widely across the mammalian body. As proof of this difference, the elastic moduli, or Young's moduli of different tissues have been characterized *in vivo* or *in vitro* by using different techniques. It was measured that the range of modulus values varies over a factor of 10^9 between the smoothest and the toughest tissues (Table 5.2).

Table 5.2: Young's modulus of human tissues assessed by different techniques.

Tissue	E (MPa)	Method	ref
Bone	18×10^3	Compression (ex vivo)	[27]
Cartilage	1.2	Compression (ex vivo)	[28]
Skin	421×10^{-3}	Rheology (<i>in vivo</i>)	[29]
Muscle	18×10^{-3}	Magnetic resonance elastography (<i>in vivo</i>)	[30]
Brain	240×10^{-6}	Aspiration device (<i>in vivo</i>)	[31]

ECM biomacromolecules provide mechanical support to the tissue structures but more importantly, the ECM provides physical anchors for cell attachment. As a result of the diversity of the ECM composition, cells evolve in a system composed of variable chemical and biological stimuli. Through cell receptors, cells can sense and

interact with different ECM biomacromolecules. Since tissues exhibit diverse mechanical properties, researchers have recognized the need to expose cells to environments with appropriate stiffness, deformation, or viscous properties to mimic the native environment. It is now well accepted that the ECM mechanical properties play a major role in the fate and function of the cells. It is well known that apart from soluble signaling, including GF or chemokines, mechanical properties of the cell-substrate can also direct cellular differentiation and movement (migration) [32]. There are several strategies to reproduce this complex environment for biofabrication of tissue models or implants.

5.4 Natural ECM for biofabrication

Given the complexity of the ECM, using naturally derived ECM-based biomaterials is a compelling strategy to provide an accurate cell culture substrate. One way to achieve this is to isolate *via* decellularization the useful biomaterials of the ECM. From allogeneic or xenogeneic natural tissues, cells are removed leaving behind the natural ECM composed of various biomacromolecules (Figure 5.3).

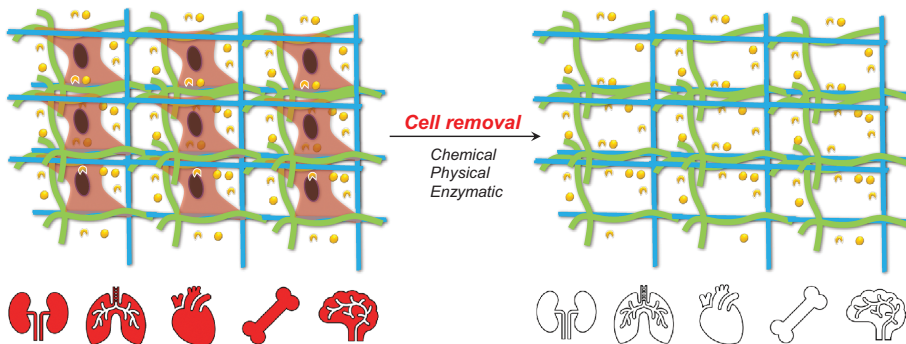


Figure 5.3: Cell removal from mammalian tissues to obtain DNA, RNA, and cell-free biomaterials. The decellularization can be carried out on full organ or tissue biopsy.

The ratio of each biomacromolecule varies depending on the type of tissue and the species from which it originates. The decellularized matrix can then be repopulated with the patients' cells *in vitro* or directly implanted into the host wherein the cells migrate and repopulate the empty matrix. Various types of decellularized organs are under development, among them, pancreas [33], liver [34], trachea [35], heart [36], cornea [37], or kidney [38] have been reported. Despite the apparent simplicity of this method, a complicated bioengineering process is required to remove the cells and all their components (e.g., DNA, RNA) without deteriorating the native ECM. To achieve this, chemically-based processes using a detergent like triton

X-100 or sodium dodecyl sulfate are used. Alternatively, enzymatic degradation with trypsin or nuclease can be used [39]. But these methodologies suffer from several drawbacks. In particular, the need for an organ donor, potential immune responses due to the use of cross-species biological materials caused by remaining genetic material, loss of mechanical properties, and difficulty in obtaining detailed characterization of the matrix materials.

5.5 Synthetic ECM for biofabrication

As an alternative to decellularization, a synthetic hydrogel that reproduces the function of the ECM: mechanical support, adhesion, and communication media can be used (Figure 5.4). Several polysaccharides forming hydrogels such as alginate or agarose have been studied and proteins such as gelatin have been brought to use as synthetic ECM. But naturally, most of the synthetic hydrogel-forming macromolecules do not reproduce cell adhesion or matrix degradation. Therefore, modification of these hydrogels is needed to accurately reproduce ECM function. For applications in biofabrications, these hydrogels must be processable, by 3D printing for example, and alginate and methacrylated gelatin are the two main molecules of choice.

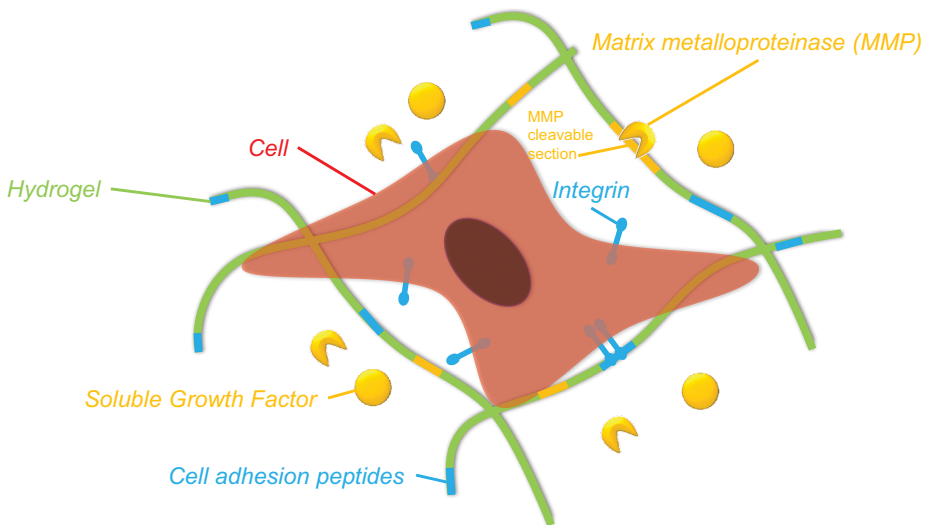


Figure 5.4: The cell in its microenvironment, showing the interactions of the macromolecules of the ECM with the cell and soluble growth factors.

However, inducing cell adhesion in alginate or MMP's induced cleavage, modification of the macromolecule is needed. To induce cell attachment different strategies can be utilized. Full-length ECM proteins when extracted from animals can be used for directing cell-based processes. But These ECM proteins are associated with certain disadvantages such as cost and potential immunogenic response. These disadvantages can be avoided by the application of short-cell recognition peptide motifs derived from macromolecules and these short peptides can be manufactured synthetically. The cell adhesion peptides (CAPs) are usually designed for a specific receptor molecule and permit to interrogate a specific adhesion pathways. Synthetic hydrogels have been modified with a variety of CAP reproducing macromolecules such as collagen, laminin, fibronectin, and elastin. These cell-binding peptide motifs mimic the signaling provided by natural ECM biomacromolecules and can direct the cell fate in a synthetic cell microenvironment. The peptide motif containing arginine (R), glycine (G), and aspartate (D) is the most prominent integrin-binding adhesion sequence and is found in fibronectin. Since RGD binds to a variety of integrins, RGD based peptides are generally applicable across most biomaterial applications. In addition to cell adhesion, cleavable peptides can be introduced in the hydrogel matrices. The crosslinking of hydrogel with the GGPQG↓IWGQK peptide cleavable by the MMP1 allows cells to remodel their synthetic environment. In turn, the introduction of a cleavable sequence upon cell generates a feedback loop where the mechanical properties of the matrices are softened much like the natural ECM. But designing this advanced system can quickly become expensive as longer peptide sequences are more expensive to synthesize . Furthermore, such advanced systems can add much complexity and the advantage of using simple systems based on synthetic hydrogen is lost. Thus, such complex systems compete with natural ECM in term of cost and model.

Table 5.3: Cell-adhesion peptide (CAP) motifs used in synthetic systems to mimic the ECM cell adhesion and to mimic ECM degradation. The arrow indicates the cleavage site.

	Macromolecule				Matrix degradation	
	Collagen I	Laminin	Fibronectin	Elastin	Collagen IV	MMP1
<i>Peptide motif</i>	GFOGER DGEA	YIGSR IKVAV	GRGDS PHSRN	VAPG	GDR GRD	GGPQG↓IWGQK

Combining different CAPs with hydrogel-forming biomaterials of different stiffness permits to elaborate libraries of stiffness and adhesions to recreate several tissue environments. These materials form then the basis for several biofabrication processes depending on their compatibility with manufacturing techniques.

5.6 Quiz



1. What is the most abundant macromolecule of the extracellular matrix (ECM)?
2. What are the two main types of polymer composing the ECM?
3. What are the main roles of the ECM?
4. How are decellularized extracellular matrices made?
5. What is a cell-adhesion peptide?
6. What is the most used cell-adhesion peptide?
7. What is homeostasis?
8. What molecules can degrade the ECM?
9. Which molecules can stop the degradation of the ECM?
10. Which molecules can be found in solution or bound to the ECM?

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6 Surface functionalization and topography for biofabrication

6.1 Surfaces for cell culture

Typical cell culture protocol involves the isolation of cells from living tissues. This requires to digest the extracellular matrix (ECM) with enzymes degrading collagen and other proteins. Once digested, the cells are free from the ECM and are collected in a container. While some cells, typically from the blood circulation, can be cultured in suspension, most of the cells need adhesion to survive. But now the cells free of ECM need to have a substrate to attach to. For a surface to provide adhesion, it must allow for adsorption of serum proteins. The mechanism of cell adhesion is a two-step process. First, proteins from the serum, supplied in the cell culture media, must deposit on the substrate's surface. This adsorbed protein cocktail can be diverse but it mainly is composed of albumin and fibronectin. When cell culture media is added into the container, the surface is brought into contact with these proteins which start to form a thin coated layer on the substrate. Simultaneously, the cells are falling by gravity at the bottom of the container and will recognize the adsorbed protein forming the coated layer (Figure 6.1). To achieve a proper coating, the tissue culture substrate must capture the protein and not alter their folding. If the protein folding is altered, it might not be recognized and thus not binding with cell receptors such as integrins which in turn will not trigger cell adhesion mechanism. However, Once attached, cells will spread and move on the surface.

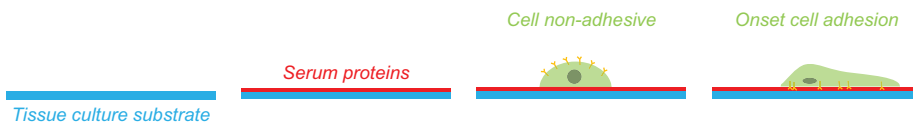


Figure 6.1: Mechanism of cell adhesion on surfaces. First, the proteins contained in the serum form a thin layer on the substrate through adsorption. Cell meets the layer of proteins and upon recognition of cell-adhesion motifs of the protein will start to spread and adhere.

It has not always been as trivial as it is today to grow cells on surfaces. Indeed, pioneers of cell cultures had only glass containers to grow the precious cells that they had harvested. The glass containers due to their brittleness could break and had to be cleaned and sterilized after each experiment. The typical sterilization process in laboratories was to autoclave the glass containers. With the development of the plastic industry, the availability of inexpensive non-brittle containers that could be supplied sterile and ready to use was a breakthrough in increasing the safety, and the reproducibility of cell-culture protocols. Indeed, one-time-use containers provided a

sterile, free of virus, germs, and fungi container. This was made possible through several developments. In the early 1960s, it was first hypothesized that poly(styrene) (PS) surfaces treated with sulfuric acid could be transformed into a cell adhesive surface. However, sulfuric acid treatment can be difficult to bring to use in an industrial setup because the toxic liquid must be then removed from the container and washed away. In the 1970s, it was then demonstrated that plasma treatment of PS can replace the sulfuric acid treatment to transform the plastic surface into a cell culture substrate.

6.2 Surface plasma treatment

Plasma is the so-called fourth state of the matter. A gas brought under a vacuum at room temperature is ignited into plasma by inducing an electrical current. This high-energy state creates charged gas molecules that can modify the surface of PS. Using oxygen as the plasma gas precursor introduces oxygen atoms onto the PS surface. As a result, the PS surface becomes less hydrophobic, and the wettability of the surface increases (Figure 6.2). This means that the contact angle between a droplet of water and the PS surface reduces as the amount of oxygen introduced on the surface is increased. This increased wetting of the surface enable proteins to adsorb on the surface and to form the required thin coating of protein necessary for cell adhesion.

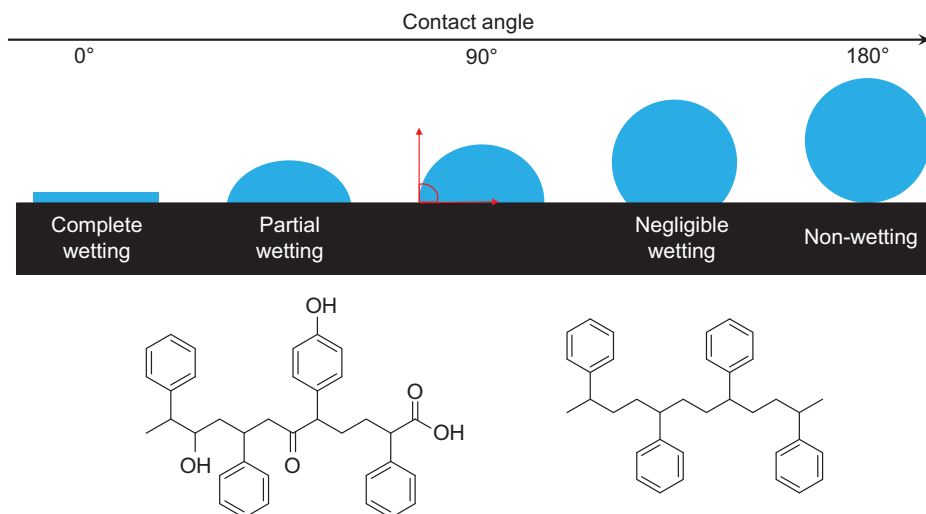


Figure 6.2: Contact angle and wettability. Air plasma treatment introduces oxygen-based functional groups on the poly(styrene) surface thus modifying its wetting capability from being negligible wetting to partial wetting.

To treat the surfaces with plasma, a dedicated setup is required (Figure 6.3). This consists of a sealed chamber into which the surface to be treated is inserted. The chamber is then brought under vacuum and different gas can be introduced into the chamber using a second inlet. Once the setup is ready, energy is provided to the system using a radiofrequency generator or current generator connected to the electrode. The stage on which the substrate is placed is grounded and the plasma can be ignited.

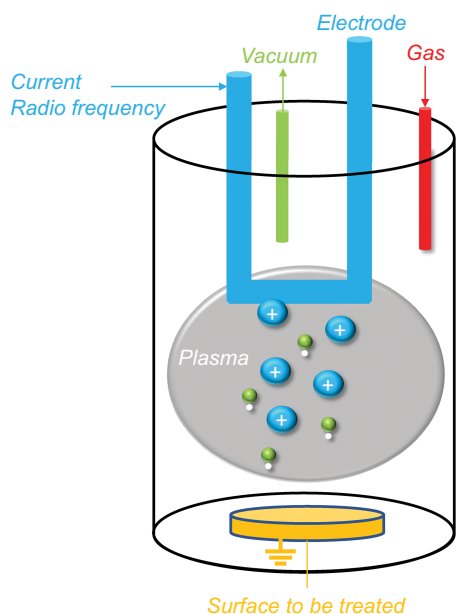


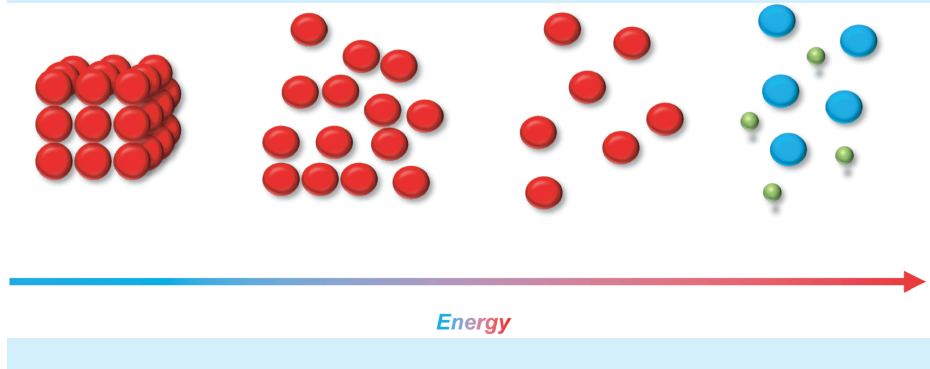
Figure 6.3: The plasma setup consists of an electrode that provides energy by current or radiofrequency into a sealed chamber. The substrate is placed into the chamber and gas can be introduced at a controlled flow rate using a vacuum. The ignition of the plasma leads to a surface modification.

The plasma once ignited gives a typical plasma glow that can vary from grey, blue for oxygen to a bright pink for argon. Using different gas for the plasma precursor will change the chemistry of the treated surface. For instance, plasma generated with a nitrogen high content gas will lead to more nitrogen atom being introduced on the exposed surface.



6.A Plasma

Increasing the energy from solid, liquid, gas and plasma the fourth state of matter. As a higher energy state than gas, plasma is an electrically conductive state. As such, long-range electrical and magnetic fields dominate the behavior of the ionized particles constituting the plasma.



6.3 Plasma polymerization surface coating

Going further with the plasma treatment, one can introduce in the vacuum chamber a monomer that has a low boiling point, so that under vacuum the monomer is introduced as a gas in the sealed chamber. Monomers with a high boiling point can be heated to allow their vaporization. Once the chamber is filled with the monomer, the gas is ignited into a plasma. The high energy of the plasma will generate radicals and charged monomers. The radical species generated by the plasma polymerize as they would do in a typical radical or anionic polymerization reaction. The monomers start to polymerize and the molecular weight increases forming on the substrate's surface polymers chains. As the reaction continues, a thin polymer coating is formed on top of the substrate made of the polymerized monomers (Figure 6.4).

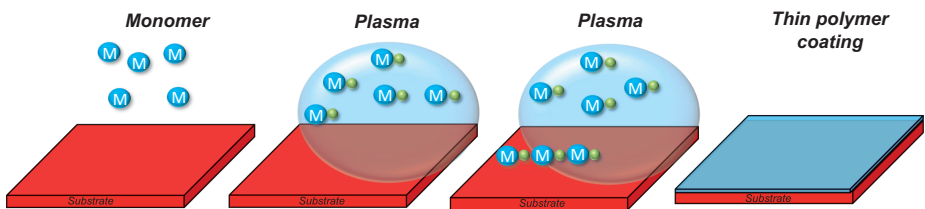
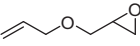
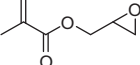
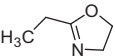
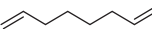


Figure 6.4: The plasma polymerization process. Where a volatile monomer is introduced into the sealed chamber plasma reactor. The monomer is ignited into plasma and it starts to polymerize. As it polymerizes, a thin polymer coating is created at the surface of the substrate.

Variation of the reaction time and monomer's flow allows for precise control of the polymer coating. Typical coating height range within 100s of a nanometer. The characterization of the surface chemistry is usually conducted with X-ray photoelectron spectroscopy (XPS) or Fourier-transformed infrared (FTIR). Several monomers have been used for the surface coating of the substrates for biofabrication purposes (Table 6.1). One of the main uses of plasma polymerization is to modify the hydrophobicity of the substrate to modulate the cell adhesion on the substrate. For instance, the incorporation of hydrophilic functional groups using ethanol and acrylic acid permit to increase the surface's wettability. Conversely, using perfluorooctane increases the hydrophobicity and gives a fluorinated coated like PTFE. Other modifications using functional groups such as glycidyl ether gives surfaces that can be reacted with amine functional groups. Allyl amine monomers are used when a surface that can react with carboxylic acid groups is needed. The main challenge in obtaining reactive functional groups with plasma polymerization is that the plasma process involves a high energy state of the matter. In this state, all kinds of active species are generated from ions to radicals, which can then react with the functional moieties rendering the final surface functionalization inert. It is thus, a matter of careful

Table 6.1: The monomers used in plasma polymerization with their associated surface coating chemistry.

Monomer	Chemical formula	Surface functionality
Acrylic acid		Carboxyl
Allyl alcohol		Hydroxyl
Ethanol		Hydroxyl
Allylamine		Amine
Allyl glycidyl ether		Epoxy
Glycidyl methacrylate		Epoxy
Alkylloxazoline		Oxazoline
Alkylamine		Amine
Propanal		Aldehyde
1,7 Octadiene		Alkyl
Perfluoro octane		Fluoro
Propanethiol		Thiol

optimization that is needed to not give too long reaction time and energy during the coating phase to not compromise the targeted reactive species [1]. For instance, glycidyl ether function is highly reactive and is thus polymerized by giving a pulse of energy to avoid the total reaction of the epoxy groups during the coating [2].

With a constantly growing library of monomers that can be used for plasma polymerization coating, a great range of applications are being developed [3]. Such modifications are easily scalable for industrial processes without the need to modify the properties of the bulk material such as the mechanical properties and resistance to constraints. These processes lead to surfaces that have antibiotic properties, can select cell adhesion, or even incorporate drugs or active molecules that can be later released once implanted or used *in vitro* [4]. These releasing surfaces are made by sandwiching the active ingredients between two layers of the plasma polymer coating. Then, the top layer wettability will regulate the release profile of the active ingredient. The higher the wettability of the surface, the quicker the ingredient is released. Complex surfaces can be achieved by using masks that can create a pattern of functional groups and gradients of functions [5]. One great advantage of plasma polymerization is that the functionalization of the surface occurs in the gas phase. This means that an intricate surface can be coated. This was demonstrated with a 3D printed porous scaffold and fibers mesh [6]. The gas will penetrate all the pores and void volumes between the fiber mesh. Upon ignition of the plasma, the polymerization occurs and the surfaces that would not be accessible with a solution can be reached. In comparison to the coating or functionalization of a surface with wet techniques, washing steps and rinsing of the substrate are not required, which has tremendous advantages for industrial processes as it greatly simplifies the functionalization method.



6.B X-ray photoelectron spectroscopy

To identify elements, present on a surface in a non-destructive manner, X-ray elemental and functional composition photoelectron spectroscopy (XPS) is one of the best methods. It is based on the photoelectric effect. For analytical purposes, it is an extremely powerful method as it gives information on the element but also to which another element they are bound to. Therefore, functional groups can be identified with XPS. The test substrate is placed in a vacuum and is irradiated with a beam of X-ray. The photon interacts with the matter ejecting electrons from the substrate and their kinetic energy is measured. From this information, spectra of binding energy are calculated, and information on the electronic shell and binding of an element is obtained. Electrons engaged in a covalent bond will have a different energy, and thus a precise map of the material composition can be obtained (Figure 6.5).

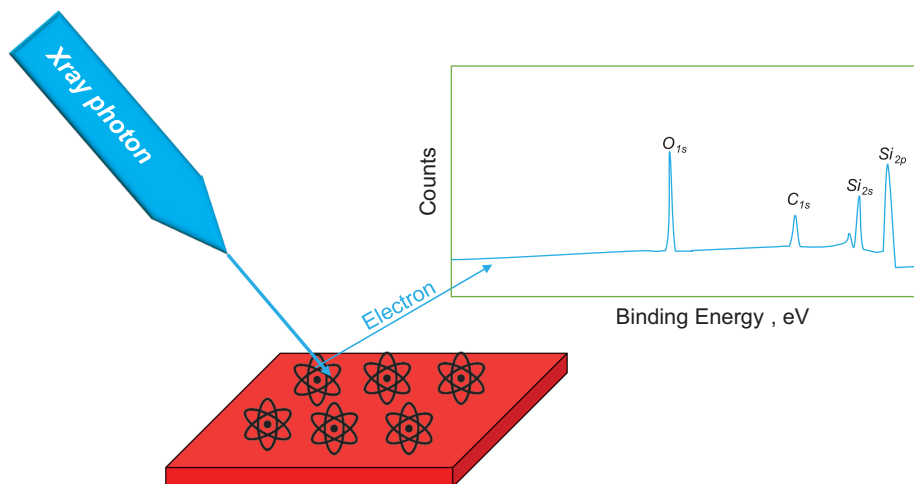


Figure 6.5: Principle of X-ray photoelectron spectroscopy. Photons are bombarded on the surface to be analyzed. The energy of the electrons ejected from the surface will provide a binding energy profile. This results let precisely analyze the chemical composition of the surface.

6.C Fourier-transformed infrared spectroscopy

The infrared principle utilizes the capacity of molecules to absorb specific frequencies that correlate with their structure. In a molecule, the atoms vibrate. Two atoms of carbon bound together will vibrate differently than an atom of hydrogen bound to an atom of oxygen. Shining an infrared light through the sample, if the light frequency is equal to the frequency of vibration of said two atoms of carbon bound together, the molecule will absorb the radiation. So, looking at the radiation passing through the samples will show a missing radiation corresponding to the vibration frequency of two carbon atoms bound together (Figure 6.6). Now, if instead of looking at one frequency at the time but all frequencies at the same time,

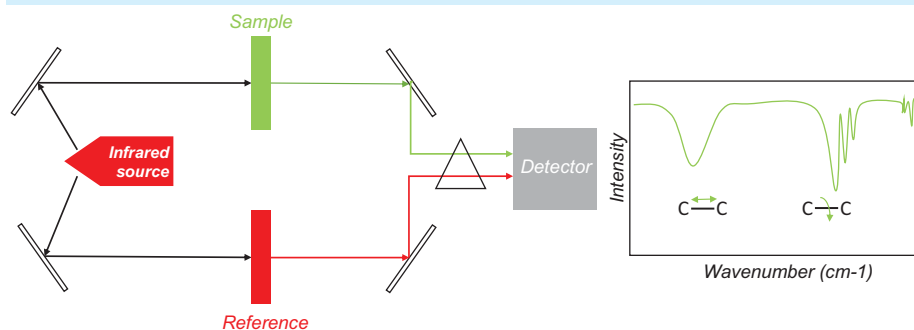


Figure 6.6: Principle of Fourier-transform infrared spectroscopy. This permits to investigate the covalent bonds.

an interferometer is obtained and is transformed by the Fourier function to obtain a frequency distribution. The signal obtained is an interferogram that contains all the frequencies that have been let through. The mathematical operation of the Fourier transforms permits decoupling the interferogram and obtaining the wavenumber spectra.

6.4 Photolithography to create shapes on surfaces

Originally developed for microprocessor manufacturing, the photolithography process has moved into the biomedical space to enable the fabrication of precisely shaped surfaces. The process consists of using wafers that are made of silicon with a layer of silicon oxide. Using a mask and a photoresist, we can selectively expose the silicon oxide layer to be etched and removed to create nanometer resolution precise surface topography. Wet etching is a material removal process that uses liquid chemicals or etchants to remove materials from a wafer. The specific patterns are defined by mask made of a photoresist that is deposited on the wafer. Materials that are not protected by this mask are etched away by liquid chemicals. These masks are deposited on the wafer in an earlier fabrication step called lithography. The whole process consists of four steps (Figure 6.7). First, a viscous resin that can be photocrosslinked is spin-coated on the silicon wafer. Then, a mask is applied above the surface of the coated wafer. The whole object is exposed to UV light. The typical photoresist used for such process is the SU-8. This is an epoxy-based negative photoresist: which means that the parts exposed to UV become crosslinked, while the unexposed part remains soluble. The SU-8 consists of eight epoxy groups that can crosslink through UV activation of photoacid: hexafluoro antimononic acid which in turn protonates the epoxides groups. Unmasked areas are activated by light and then polymerized during the baking step. The uncrosslinked photoresist (the part masked from the UV) is then washed made of. The photoresist protects the silicon wafer below it from the etching solution of hydrofluoric acid which remove the silicon from the wafer. Different types of etching can be conducted: isotropic (uniformly in all directions) or anisotropic etching (uniformity in a vertical direction). Some of the anisotropic wet-etching agents for silicon are potassium hydroxide (KOH), ethylenediamine pyrocatechol, or tetramethylammonium hydroxide. Etching a silicon wafer would result in a pyramid-shaped etch pit. The etched wall will be flat and with an angle. For isotropic wet etching, the most common agent is a mixture of hydrofluoric acid, nitric acid, and acetic acid. As the reaction takes place, the material is removed vertically and laterally at the same rate. Once etched, the photoresist is removed to reveal the pattern surface. This technique permits to create nanometer-scale structures and surface topography at high precision. However, dry etching, which followed the same workflow, is much more precise. Usually, dry etching is done using a plasma reactive gas such

as fluorocarbons, oxygen, and chlorine with an addition of other inert gases like nitrogen or argon.

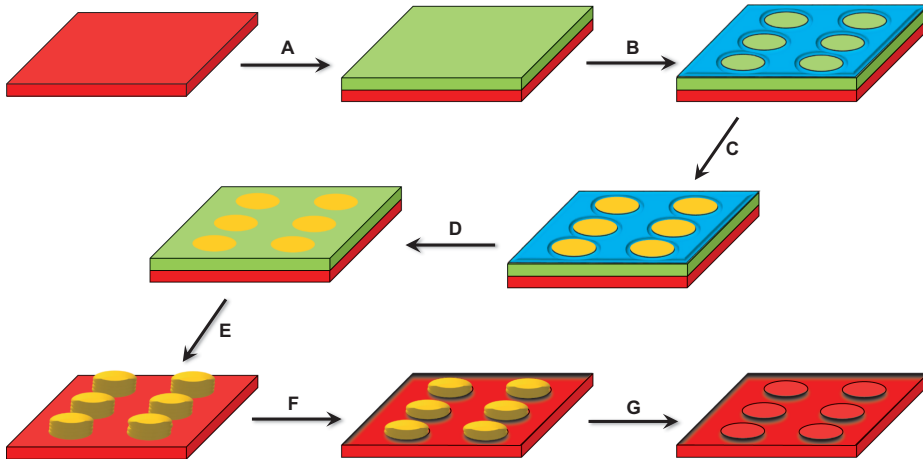


Figure 6.7: The photolithography process: (A) The silicon wafer is coated with a photoresist, (B) a mask is applied onto the surface, (C) the wafer is exposed to UV light (D) crosslinking only exposed area. (E) uncrosslinked photoresist is washed away exposing a (F) bare silicon wafer that can be etched. (G) Finally, the crosslinked photoresist is removed.

6.5 Nanopillar and nanoneedles

The photolithography etching method described above has one drawback, that is, the etching of the silicon wafer does not give a straight angle. Typical wet etching using SU-8 gives a pyramidal etched pattern with a 54.5° .



Figure 6.8: (left) Wet etching using a photoresist giving a pyramidal topography versus (right) metal-assisted wet etching giving a straight etching.

If we want to get straight etching, a more conductive layer is needed. This process is called metal-assisted chemical etching which can be obtained by adding a metal layer on top of the photoresist to coat the wafer with a metal such as silver or gold and masking the metal with PS beads (Figure 6.8). Several variation of these methods have been developed over the years using various metal coating and different masking

techniques. The metal coating catalyzes the etching of the silicon to obtain an anisotropic process that allows the fabrication of pillar. These Si pillars have many applications in modern electronics ranging from sensors, batteries, and solar cells (Figure 6.9).

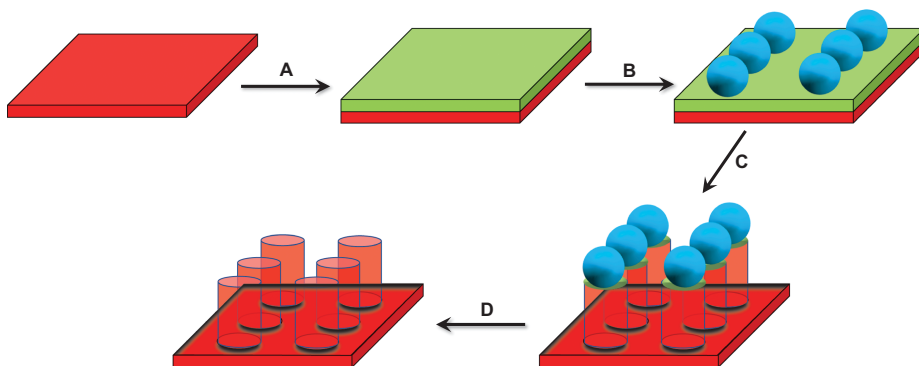


Figure 6.9: The metal-assisted chemical etching process (MACE). (A) A silicon wafer is coated with a metal layer, (B) an isolator such as poly(styrene) beads can be used to form the patterns, (C) chemical wet etching occurs in an isotropic manner giving pillar or wires, and (D) the PS beads are dissolved.

Beyond their applications in electronics, nanowires and nanopillars have been proposed for biomedical applications [7], for example, as a research tool to interrogate the traction force of cells. Cells culture on the pillar will deflect the pillar to a certain angle depending on the traction force applied to the pillars. Measuring the deflection angle permits the measurement of the cell traction forces (Figure 6.10) [8].

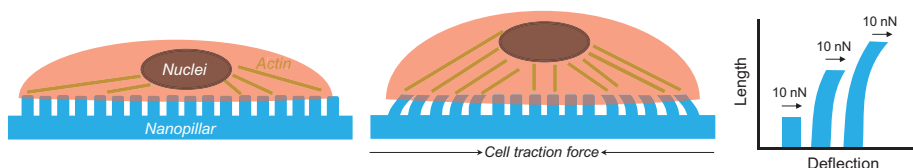


Figure 6.10: Silicon nanopillar used to measure the traction forces involved during cell adhesion and movement by measuring the deflection of the pillar.

Further development of the etching technique has led to the manufacturing of nanoneedles, that is, pillars that are pyramidally shaped and which can pierce the cell membrane for transmembrane delivery [9]. By creating an array of these nanoneedles, drug delivery devices can be made. Intending to replace the often-painful needle injection, needle array permits to multiply the surface area to deliver the same amount of drug through the skin and reduce the pain. Different types of needles have been proposed such as coated needles that leave the active ingredient in

the skin once removed (Figure 6.11). Other design can come as a solid needle that pierces the skin to allow deposition of the drug. Further needle design consists of a porous or breakable needles that once implanted are left behind and degrades over-time to release their active pharmaceutical ingredient (API). More complex hollow needles act as a classical needle through which the API is pushed into the skin. Further applications of these nanoneedles array are for constant implantation and monitoring and sensing of physiological and biochemical markers [10].

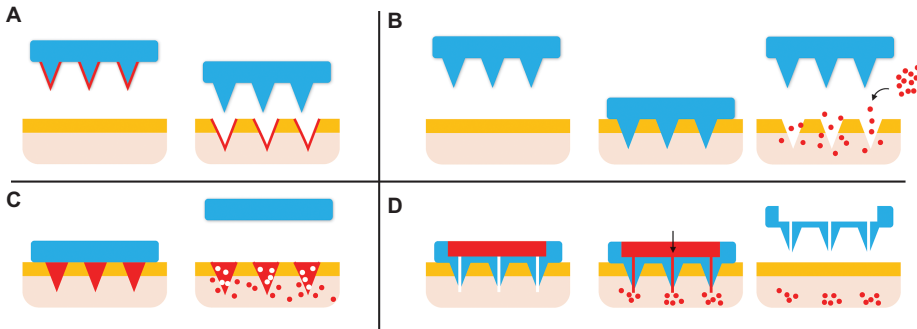


Figure 6.11: Different types of nanoneedles array technology proposed for drug delivery: (A) Coated microneedle, (B) solid removable nanoneedle, (C) dissolving microneedles, and (D) hollow microneedle.

6.6 Soft lithography

Compared to photolithography, soft lithography is a process that instead of using a photoresist to create a topology, is using an elastomeric stamp to create the surface topography. The most common materials to make the stamp is poly(dimethylsiloxane) (PDMS) due to its biocompatibility, viscosity, chemical versatility, and low surface tension. Soft lithography is a family of techniques for fabricating surface topology that is then used to sort cells, create synthetic organs on a small surface called organ-on-a-chip (OOC) [11, 12].

6.6.1 Replica molding

When talking about soft lithography the first method that comes to mind is the replica molding technique. This is by far the most common technique (Figure 6.12). Starting from a master mold that is made out of a silicon wafer manufactured through photolithography or other manufacturing technique, PDMS viscous solution is then poured on top of the master mold. The PDMS is cured (crosslinked) and peeled off the mold giving a negative replication of the master mold. The PDMS mold can then

be replicated after being rendered non-adherent through silanization. This PDMS stamp can be used several times. The replica molding process is usually made with PDMS, but other soft materials have been used. Hydrogel made of gelatin methacrylate or agarose is also a good candidate for this technique which is particularly suited for making microwells.

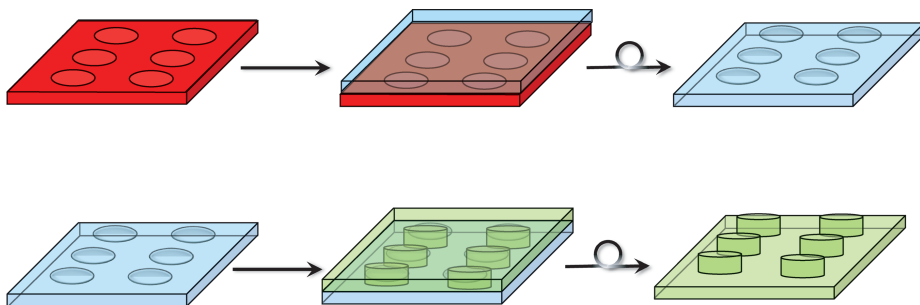


Figure 6.12: Replica molding technique. A master mold (red) is used to make a negative PDMS stamp (blue). The stamp can be used several times to stamp another PDMS or hydrogel (green).

6.6.2 Capillary molding

A technique particularly suited for the fabrication of small linear patterns is the capillary molding method (Figure 6.13). Using a PDMS mold with capillary size channels applied to a substrate, a polymer precursor is sucked into the channel through capillary force. This pre-polymer solution can be poly(ethylene glycol), PDMS, or hydrogels such as gelatin methacrylate and agarose. The polymer is cured and the polymeric mold is peeled off giving a structured polymer microstructure. This sort of patterning is interesting when cells need to be aligned on a substrate or guided through a linear pattern, for instance to replicate the architecture of blood vessels [13].

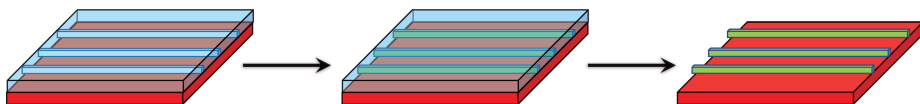


Figure 6.13: Capillary molding using a PDMS mold (blue): a pre-polymer solution (green) is infused into the channel of the mold. After curing, the mold is removed and the polymer stays on the substrate (red).

6.6.3 Microcontact printing

Printing of proteins or peptides motifs on a precise pattern on a surface can be made with a PDMS stamp (Figure 6.14). The stamp has a protrusion that has ink on the surface. The stamp is inverted and brought in contact with the substrate and the molecule of interest is either adsorb on the substrate or covalently bonded. Typical chemical reactions are with an amine of the biological molecules that can react with an epoxy-functionalized surface (for instance, plasma coated surfaces). Different shapes can be made, and these can be used to study how cell adhesion occurs on these coated surfaces [14]. Other application of this microcontact printing is particularly useful for high-throughput screening where a high density of environment is to be tested. The proteins on the stamp can be varied thus allowing a rapid fabrication of test surfaces for cell adhesion [15].

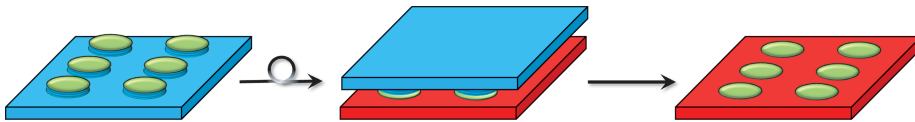


Figure 6.14: Microcontact printing the transfer support is covered with ink. Inverted and stamped on the substrate.

6.6.4 Microtransfer molding

A stamp can also be used to transfer a polymer on a surface (Figure 6.15). The polymer precursor solution is loaded on the microwell of the PDMS stamp. The stamp is inverted and brought into contact with the substrate. The polymer solution is cured and the stamp is peeled off leaving a pattern of cured polymer. A typical polymer that can be used is PDMS, but also hydrogels such as gelatin methacrylate, collagen, or agarose. Into the patterned hydrogel, a cell can be cultured within the confined space of the hydrogel. This permits a reduction of the size of the cultured sample and forces the cells to organize into a defined shape [16].

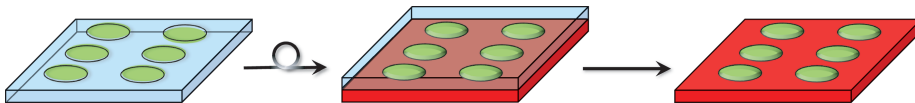


Figure 6.15: Microtransfer molding precursor polymer is loaded in the well of the transfer support. The support is inverted, the polymer is deposited on the substrate, cured, and the transfer peeled off the substrate.

6.7 Microfluidic devices

6.7.1 Laminar flow

With all the techniques available for surface functionalization and fabrication such as plasma functionalization, photolithography, silicon etching, and soft lithography, complex microfluidic devices can be designed and manufactured. One of the particularities of a microfluidic chip is the small channel diameter that is used to reproduce the blood flow. Using a smaller diameter channel, the fluid will be under high shear flow and will have a laminar flow meaning that the fluid will difficulty mix (Figure 6.16). The small diameter used for these OOCs permits to reduce the volume of liquid needed, thus reducing the cost of development by using a minimum amount of reagents and cells.

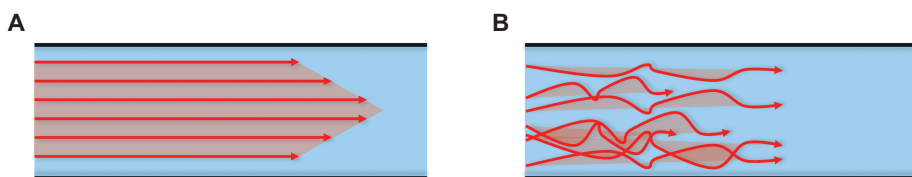


Figure 6.16: Schematic of (A) laminar flow in microfluidic channels as compared to (B) turbulent flow.

This laminar flow in small channels can be leveraged in chemistry to build small reactors and simulate industrial setup [17, 18]. When a fluid is flowing through a closed channel, two types of flows may occur depending on the velocity and viscosity of the fluid: laminar flow or turbulent flow. Turbulent flow is characterized by rough flow where mixing of the fluid can happen. Laminar flow is characterized by smooth flow where the mixing of components is mainly driven by diffusion, a slow process.

The Reynolds number quantifies the relationship between fluid viscosity and fluid velocity and allow for the calculation of a threshold between the turbulent and laminar flow. The Reynolds number, that is, the values where laminar flow occurs, will depend on the geometry channel and flow. In a microfluidic channel, the Reynolds number is defined as

$$\text{Re} = \frac{\rho u D_H}{\mu} = \frac{u D_H}{\nu} = \frac{Q D_H}{\nu A}$$

with D_H for the hydraulic diameter of the channel (m); Q for volumetric flow rate (m^3/s); A for the pipe's cross-sectional area (m^2); u for mean speed of the fluid (m/s); μ for dynamic viscosity of the fluid ($\text{Pa} \cdot \text{s}$); ν for kinematic viscosity of the fluid (m^2/s); and ρ for density of the fluid (kg/m^3).

6.7.2 Cell sorting

The laminar flow easily reached in microfluidic devices can be leveraged to create cell sorting devices with a small surface ratio. Different chip designs with either passive or active cell sorting capabilities have been proposed. Using the laminar flow, cells can be sorted out by size in the channel. An array of posts can also be used to sort cells by size by disrupting the flow of cells [19] (Figure 6.17).

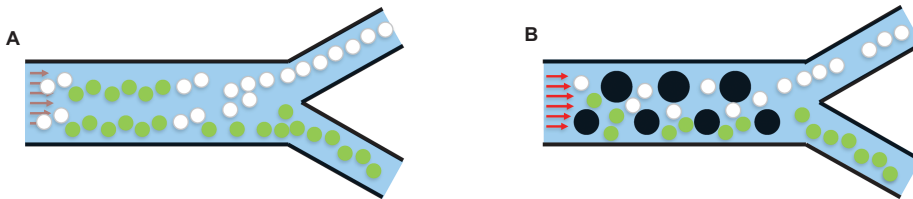


Figure 6.17: Microfluidic cell-sorting device capable of sorting the cell by size. The bigger cells (green) are separated from the smaller ones (white). (A) By laminar flow and (B) using post that disrupt the flow.

But not all cells can be sorted based on their size. When a cell population must be sorted based on their protein expression, more complex devices that rely on an active substrate are needed (Figure 6.18). Microdevices that can retain cells by presenting an antibody that recognizes cell surface markers are quite efficient in purifying a cell population. An alternative technique used a laser that can recognize a fluorescently tagged cell marker that then can trigger an active sorting mechanism to direct a type of cell toward a dedicated channel. Microbeads tagged with an antibody that can selectively bind to a cell population can be useful in creating a size gradient between two cell populations to sort them in a passive sorting device. Building on microbeads, antigenic and fluorescence technologies microfluidic cell sorting devices can become extremely efficient in sorting cells on a small footprint. The emergence of this technology has been seen as having a great potential in miniaturizing biomedical devices for diagnostic purposes but also to provide affordable tools for cell therapies currently in the development pipeline [20].

6.8 Organ-on-a chip

Most of the drugs discovered in the past were done by serendipity. Case in point, the discovery of penicillin was possible as Fleming left a culture plate on a window for several days. When he came back, he found that fungi had grown and inhibited the growth of bacteria. Many other drugs have been discovered from natural compounds. Today, pharmacy is a business and as such, it cannot rely on sheer luck. Therefore,

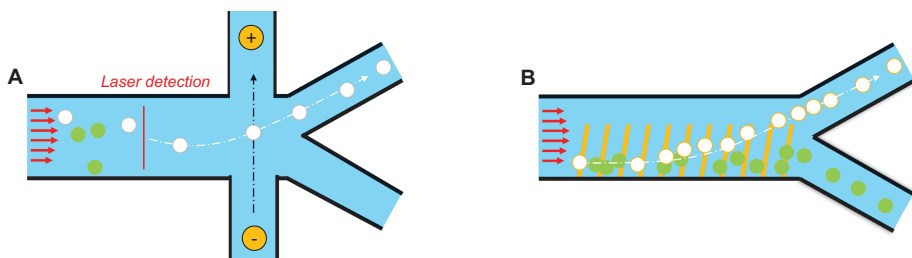


Figure 6.18: (A) Active cell sorting where a laser detects the fluorescent tag of a cell before actively sorting the cell through an electromagnetic field (if the cell is loaded with magnetic particle), or with a lateral microfluidic flow. (B) Sorting through antigen adhesion the yellow strips that guide the white cells toward the upper outlet.

methods and processes to foster the discovery of new compounds have been put in place to ensure the capability to bring new molecules to the market.

Currently, the drug discovery process starts by creating a library of molecules that you test on a cell line or cellular assay that reproduces the disease you want to target. The disease has been selected as part of an industrial decision with the marketing and medical department. You will screen the different molecules, refine the structure of the molecule using *in silico* computer methods. Once you have your molecule, you will test it *in vivo* using animal models that reproduce the targeted disease. Once your molecule passes these steps, you can start the human phase with the clinical trial. First assess the safety of the drug, then the efficacy, and finally the efficacy on different populations and monitor the side effects. But this development takes decades, but the patent on the molecule, which gives you exclusivity, expires after 20 years.

In addition to this cost, not all the molecules that are discovered make it to the market. Some molecules are not better than current treatment, some molecules are not safe leading to failure of the clinical trials. Indeed, dozens of clinical trials failed every year and sometimes led to fatality when the tested molecule is found not safe. While many molecules work on laboratory animals, many are not working in humans.

There is therefore a great interest in reducing the part of animal testing to get drug development on models that better reproduce human physiology. This would enable to earlier assess the potential of the molecules and avoid failure of clinical trials. On top of this industrial and scientific need, there is a great societal interest in removing the reliance on animals for the testing of active pharmaceutical ingredients (API). Decades ago, the cosmetic industry had successfully banned animal testing and replaced the safety testing of new molecules with tissue-engineered skin. But while cosmetics are contained to the skin, API are traveling into the bloodstream and can reach all organs. Therefore, models that reproduce human physiology and drug circulation *in vitro* are needed if we want

to reduce or replace the reliance on animal models for the commercialization of safe and efficient drugs.

Not all drugs are equal. While some drugs are injected directly into the bloodstream (intravenously), some drugs are taken orally, and this makes a huge difference. Indeed, when you inject a drug into the bloodstream, it will directly travel to all the organs. An oral drug will have to pass through the digesting track and the liver before entering the bloodstream. As the drug goes through the liver, it might be treated by the liver enzymes and modify its chemical structure or the dose that is available in the blood circulation is drastically different than the amount taken. This field of study called pharmacokinetic is a big part of understanding how drugs work and generates key data to design the molecule delivery mode, the molecule dose, and interval of injection or ingestion. Conversely, the field of pharmacy focusing on how the drug works once ingested is called pharmacodynamic. One of the best methods to assess cokinetics is to use animal models. Although all animals have physiology as humans (blood temperature in mouse 36.6 °C, in rat 35.7 °C), it can help to extrapolate the pharmacokinetics in humans. Organ-on-a-chip (OOC) approach consist of using soft lithography fabrication techniques to create model organs that uses human cells organised in a microfluidic chip. OOC, by combining different synthetic organs, is proposed to serve as a simulation of human pharmacokinetic *in vitro* (Figure 6.19) [21].

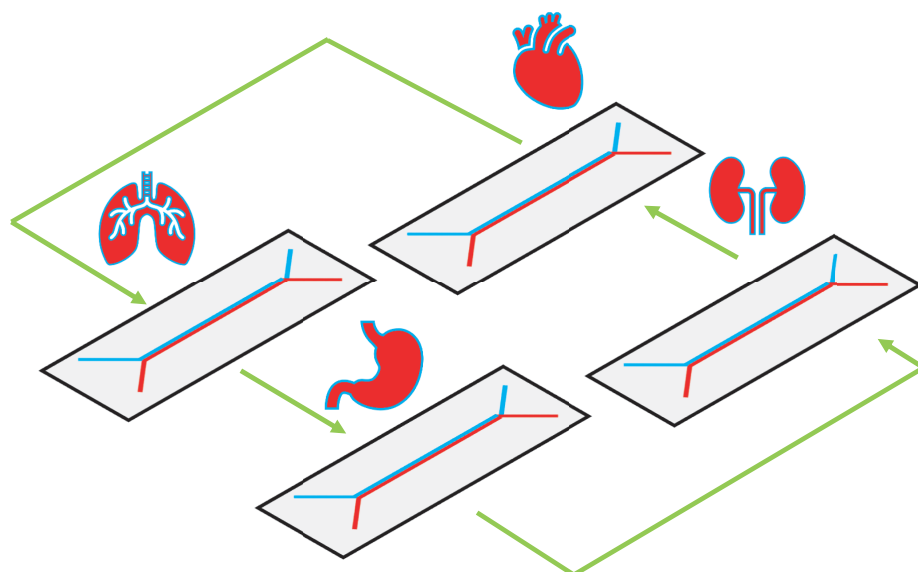


Figure 6.19: Serial assembly of microfluidic chips to simulate the human physiology and blood circulation between organs from the heart, lung, stomach, pancreas, and liver.

6.8.1 Simulated organs

When reproducing an organ synthetically *in vitro*, two strategies can be followed: reproducing the minimum required function or mimicking the design from nature. The soft lithography methods used to manufacture microfluidic chips while quite advanced suffer from many limitations that render challenging to perfectly mimic the natural architecture of human organs (Figure 6.20). Therefore, most efforts have been focusing on mimicking the key function of organs. Some organs have a tissue barrier: they protect one organ system from the other and thus serve as a selective exchange of molecules. For instance, the lung serves for the exchange of gas between the air and the bloodstream. Other organs have been mimicked on a chip. For instance, the blood–brain barrier (BBB) serves as a selective exchange hub which able to protect the brain from detrimental molecules or parasites that could have made it into the bloodstream. This can be reproduced by designing a chip with two compartments composed of an interface to mimic the BBB. Other organs, such as the intestine, can be similarly reproduced with a two-compartment system. The liver function can be mimicked by using a hydrogel containing the different types of liver cells. Mimicking the kidney function also requires a hydrogel to serve as an exchange media between arteries and veins. Each of these organs can be redesigned with a simpler approach compatible with microfluidic chip manufacturing methods [22, 23].

One of the key advantages of OOCs is the modularity and versatility of the platform. Organ modules can be brought inline or not depending on the experiment, modules can be upgraded as the technologies evolve. Finally, the microfluidic chip

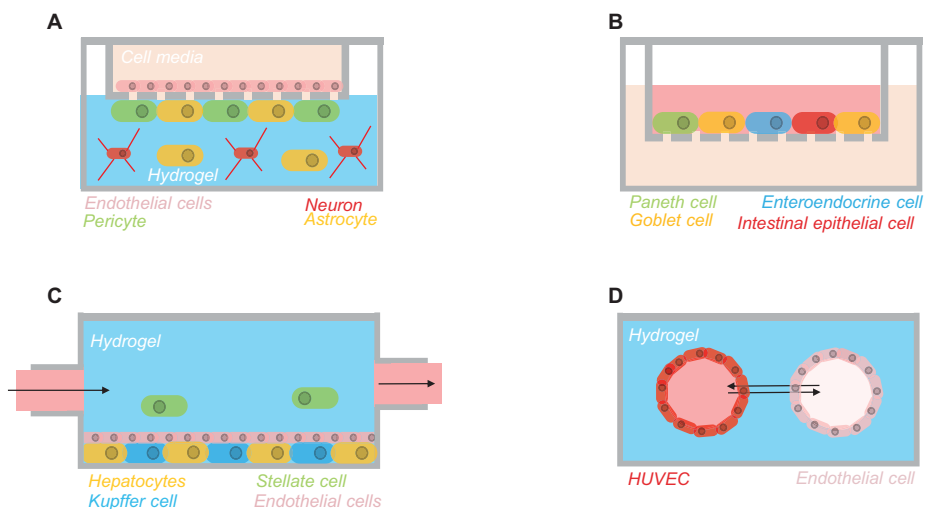


Figure 6.20: Schematic of the example of microfluidic cell models of (A) the blood-brain-barrier, (B) intestine, (C) liver, and (D) kidney.

setup can be scaled up and multiplied easily when maybe one day OOC can replicate the patient physiology *in vitro* and allow physicians to test a drug regiment before administrating it to their patients. With this potential, it is expected that OOC technology will gain much traction in the future and be a key player in our endeavor to replace animal testing for drug discovery.

6.9 Quiz

1. What is the contact angle of a non-wetting surface?
2. What are the two steps of cell adhesion on PS?
3. What is the most energetic state of the matter?
4. What is the Reynolds number useful for?
5. What is the difference between photolithography and soft lithography?
6. What is the method used to manufacture nanowires?
7. Which analytical techniques are best suited for the characterization of surface chemistry?
8. What are the applications of nanopillars?
9. Which societal and industrial needs does OOC technology promises to answer?
10. What is the difference between pharmacodynamic and pharmacokinetic?



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7 Porous scaffold

7.1 What is porosity?

In material science, porosity measures the void fraction, the empty spaces in a material. It defined the fraction of the volume that is empty over the total volume of the material as a percentage. Several techniques are available to calculate the void volume in materials. Choosing the proper method depends on the type of porosity and materials. Once the empty space of materials is measured, the porosity can be calculated as follows:

$$\varnothing = \frac{V_{\text{empty space}}}{V_{\text{total}}}$$

In nature, many materials are porous, that is, they contain empty space. A prime example is rocks such as sandstone or limestone made of pores that allow them to filter water. In mammalian, many tissues are made of a porous structure. A prime example is the bone inorganic matrix that has a porous architecture. But not all pores architectures are built equals. We must distinguish two types of porosity: the closed pores and the open pores architecture (Figure 7.1). The pores are not connected to each other in the close pores architecture, while most pores are connected in the open-pore architecture. This means that nutrients, cells, and gas can pass from one side of the porous structure to the other in the open pore architectures, while in the closed-pores architecture, the two sides of the porous architecture are not connected. Within these two architectures, pores can have different shapes. Pores can be square, rounded, or have an elongated structure, and this has to do with the way there are made.

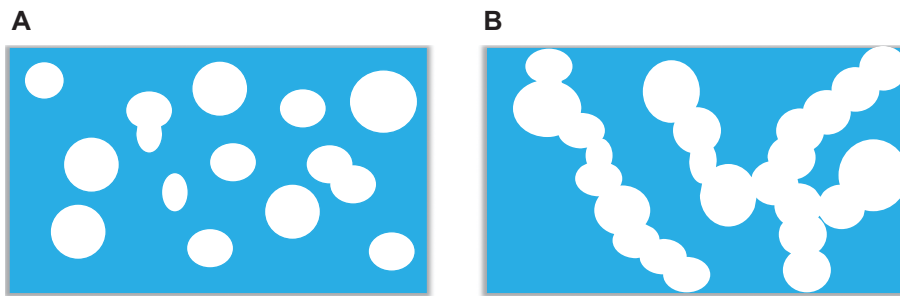


Figure 7.1: The two different pore architecture. **(A)** Closed-cell pores and **(B)** open pores.

There are several techniques to generate pores and create porosity in a scaffold or materials, and in this chapter, these different approaches are presented [1].



7.A Measuring porosity

Once a porous scaffold is obtained, several techniques can be used to measure the porosity of the scaffolds. Not all the techniques are compatible with all samples (Table 7.1). This will depend on the type of pores (closed or open pores), the size of the pores (micro- or mesopores), and the scaffold materials. Indeed, it can be extremely challenging to measure the porosity of scaffolds made of soft materials that can deform under stress. Therefore, the measuring technique must be carefully chosen to get an accurate measurement.

The pore definition from the International Union of Pure and Applied Chemistry (IUPAC) defines porous materials as being microporous when the pore diameter is less than 2 nm, mesoporous when the pore diameter is comprised between 2 and 50 nm, and macroporous when pore diameter is greater than 50 nm. In the case of porous scaffolds used in biofabrication usually, the pore size is greatly higher. Indeed, the porogen-made scaffolds have pores usually in hundreds of nanometer to micrometer scale. This great range of pore size must be taken into consideration when choosing the measuring technique.

Table 7.1: Techniques used for the measurement of porosity.

Technique	Principle	Pore type
Brunauer–Emmett–Teller (BET) theory	Gas (nitrogen) is adsorbed on the pore surface forming several layers. The pressure required to equilibrate the system provides information on the surface area.	Nanometer: micro- and mesopores according to IUPAC. The materials must resist the pressure.
Mercury intrusion	Mercury is forced into the pores under pressure. Then, the applied pressure lets us calculate the surface area and pore size.	Open-pore architecture between 250 μm to 3 nm. Specialized for hard materials needs to withstand the high pressure
Atomic force microscopy	The surface of the sample is scanned with a cantilever. The difference in surface height between pores reveals a 2D map of the surface topography.	Nanometer to micrometer open-pore architecture. Only surface pores
Scanning electron microscopy	An electron beam is shot at the surface of the sample. The backscattered electrons are captured by a receptor and give an image of the sample surface.	Nanometer to micrometer open-pore architecture of the surface porosity. Conductive and vacuum resistant materials
X-ray tomography	X-rays are used to scan the inner architecture of the sample. Each scan is computed to create digitally a 3D image of the object.	Provide a 3D image of the sample and the porosity. Depending on the resolution of the instrument micrometer, open or closed pores

Table 7.1 (continued)

Technique	Principle	Pore type
Nuclear magnetic resonance (NMR)	A medium such as water is used to probe the pore size. The capability of the media to interact with the movement of the probing media is measured.	Suitable for hydrogels with pores in the hundreds of a nanometer to tens of a micrometer.

7.2 Porogen-induced pores

One of the methods to create pores is to use a porogen. We can define porogen as a mass of particles of a specified shape and size used to make pores in molded structure, and the porogen is removed after the structure has set. These structures can, in turn, be used for different applications, from insulation foam to tissue engineering. Depending on the type of porogen, one can design and manufacture a variety of porous scaffolds.

7.2.1 Foam-based porous scaffolds

A scaffold that is constituted of small bubbles is called foam. Typically, foams in physical chemistry are defined as a colloids system constituted of gas particles dispersed in a continuous liquid medium. By extension, the term foam is applied to materials that are constituted of a cellular architecture, such as a sponge. One typical example of foam in polymer science is polyurethane foam, which combines the two monomers to make the polycondensation reaction (Figure 7.2). Upon reaction of the monomers with each other, carbon dioxide is generated. The gas bubble is generated and will then travel throughout the material, expanding the polymer matrix to create porous materials, a foam. Because we use a gas medium as porogen, the gas wants to escape in need of more space. Therefore, the gas bubbles will travel throughout the material to create an interconnected system of pores [2] (Figure 7.3).

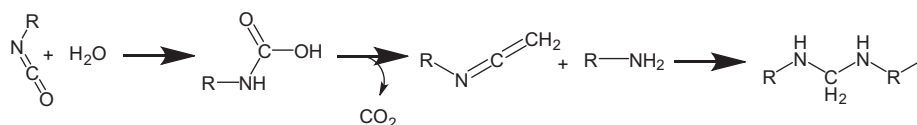


Figure 7.2: Chemical reaction of polyurethane.

In tissue engineering, open-cell materials are highly interesting as they permit the creation of materials that can reproduce natural tissue architecture. It allows nutrients, gas, and cell to travel throughout the materials. But creating sponges using gas that can be harmful to cells must be done *in vitro*, and only when the materials are cured and neutralized then cells can be added. Beyond polyurethane, other materials can be made as foam. The key here is to have a crosslinking reaction that generates a gas. If the gas is toxic, one must neutralize it and ensure that there are no more remaining toxic chemicals. But the major drawback of these foams is that it is challenging to control the size of the pores, the pore organization, and all the pores will have a rounded shape.

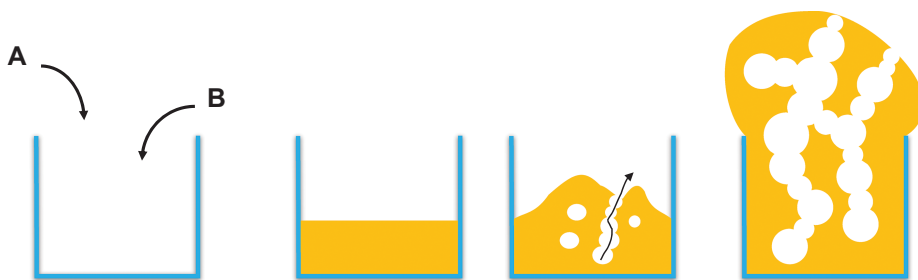


Figure 7.3: Scheme of the foaming process of polyurethane monomer A and B are mixed together in a container. The reaction occurs generating gas that travel in the polymer matrix creating the porosity.

7.2.2 Crystal-induced porous scaffold

To control the pore size of a polymer matrix in a precise manner, we can introduce a porogen of pre-determined size. Salt of NaCl or sugar that have been ground and sieved to obtain a controlled crystal size are mixed in a melted polymer matrix such as PLA or PCL. The melted mix can then be molded in a particular shape and allowed to set at a temperature below the melting temperature. The porogen, which is made of a water-soluble crystal, can be leached out of the matrix by simply immersing the object in deionized water and refreshing the water regularly to be consistently below the saturation concentration of the salt or sugar, in our example. After a few rinses, a porous scaffold is obtained. Sodium chloride (360 g/L) and sucrose (2,000 g /L) are good candidates as they have a high saturation concentration which permits rapidly leaching them out of the polymer matrix [3, 4] (Figure 7.4).

With this technique, we can control the pore size, but not the organization of the pores. In the case of a crystal sample having a broad size distribution, the physical effect of convection can be leveraged to create a gradient of particle size in the scaffold. In a sample made of different particle sizes, the bigger particles will rise to the top while the smaller ones will stay at the bottom. This phenomenon, also called the muesli effect, can be used to organize the porogen particle into a size gradient (7C).

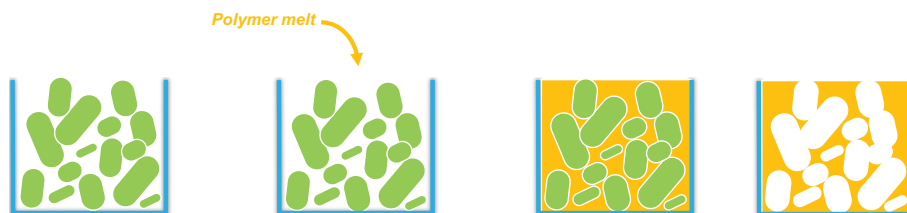


Figure 7.4: From left to right: Crystals are organized in a pattern, then the melted polymer matrix is added to the crystals, the material is set, and the porogen is removed.

When using the crystal technique, the second important consideration is that salt and sugar do not crystallize into a round shape. Since the shape of the crystal will be translated to the polymer matrix, it will govern the resulting pore shape. In our example, sodium chloride forms a face-centered cubic lattice, and sucrose forms a hexagonal prism. Using an extensive ball or roll milling can help change the shape of the crystals, but this can become a tedious process.

Similarly, to the melt process casting, the polymer can also be dissolved in a solvent that does not solubilize the porogen. The porogen is added to the polymer solution. The solvent is evaporated, and the bulk material is then immersed in the porogen's solvent. Depending on the packing of the porogen, an open- or closed-pore architecture can be obtained. If the porogen are pack in a manner that they are touching with each other, then a open-pore architecture is obtained. However, to obtain a closed-pore architecture, the vent used to remove the porogen needs to travel into the polymer matrix. Many small molecules and active pharmaceutical compounds can be crystallized. As crystals, they can be used as a porogen and incorporated into a polymer matrix. Once processed into an implant, the porogen will be slowly leached out of the polymer matrix after implantation.

7.B Convection effect

When opening a bag of mixed nuts, have you ever wondered why the pecan nuts and other larger nuts are always on the top of the bag? Why at the end of the bag do you get all the smaller pistachios? This is due to the physical effect called convection or also called the Brazilian nuts effect or muesli effect. It states that when particles of various sizes are shaken in a container, the bigger particles will float at the surface (Figure 7.5). This principle is the key to avalanche safety inflatable backpacks. These safety devices have a balloon that can inflate when needed. This balloon expands when a skier is trapped in a snow stream and increase the skier size that can make him bigger than the snow aggregates and thus will help him float at the surface of the avalanche due to the convection effect. Using the convection effect when making scaffolds with particles of different sizes, will bring the bigger particles at the top and smaller particles at the bottom. Thus, one can use the convection effect to create a gradient of particle size upon shaking container of porogen of various size. Once the particles are organized in a gradient, the polymer melt or solution can be added following one of the porogen-based fabrication techniques presented in this chapter.



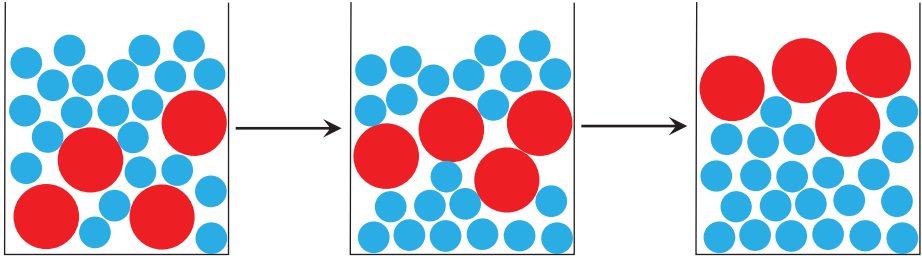


Figure 7.5: The convection effect: in a population of mixed-size particles, when shook the bigger particles float at the top of the mix.

7.2.3 Porogenic solvent

Because a porogen is a pore-generating phase, this can also come as a liquid. Solvents that are inert and can solubilize a polymer and have a boiling point that is lower than the melting point of the polymer matrix can be used to create pores. Typically, a polymer melt is mixed with a solvent. Once processed together, a phase separation must be induced to create a porous structure. Once the phase separation is obtained, the solvent can be removed by evaporation [5] (Figure 7.6).



Figure 7.6: From left to right: Solvent of the polymer is mixed with a polymer melt, then a homogenous solution is obtained, next a phase separation is generated by cooling down the matrix, and the solvent is evaporated, leaving a porous scaffold.

Different solvents can be used to create various type of pores. For instance, the use of methanol in a PLA melt leads to higher pore diameter as if a mix of ethanol and methanol is used or pure ethanol. This has to do with the solvent interaction with the polymer. A poor solvent of the polymer will give a larger pore than a good solvent because the phase separation will be more pronounced [6].

7.2.4 Water as a porogen

While few polymers are soluble in water, the case of water as a porogen medium is quite interesting. Indeed, water can be processed into a solid shape and sublime, that is, transition from the solid phase to the gas phase without becoming liquid. Present in major quantities in hydrogels, such as collagen, it can form highly porous sponges.

By depositing water droplets on a surface of known hydrophobicity, controllable water droplet size and shape can be obtained. The droplet can then be frozen. The resulting ice particles can be dispersed into a collagen solution. The ice crystals can then be recrystallized to create a connection between them leading to an open-pore architecture. Finally, the water is removed by sublimation leaving a porous scaffold (Figure 7.7). One can create precise and complex architecture by playing around with water droplet size, shape, and organization. [7, 8]

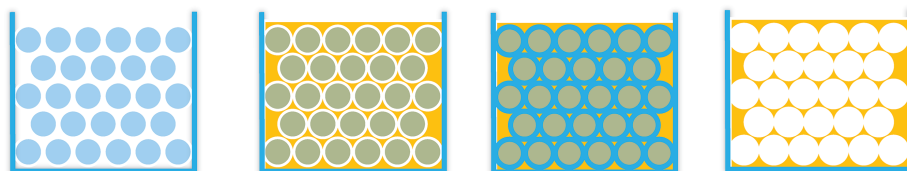


Figure 7.7: From left to right: Ice droplets are formed by freezing water droplet. These ice particles are then mixed with the polymer, and then connected with each other through a second freezing parameter. Finally, the water is sublimated, leaving a porous matrix.

In the case of hydrogels, removing the water by freeze-drying reveals the internal organization of the polymer chains. It is then possible to observe how the polymer organizes into the water media. However, one must acknowledge that the freezing process can modify the natural organization that the fibers have in a liquid phase. Nevertheless, agarose polymer chains organized into helical structure leave rounded pores when freeze-dried. Methylcellulose polymer chains that organize into a β -sheet leave a laminar porous scaffold once freeze-dried [9].

7.C Phase diagram of water

Water can be found in three different states on earth: solid (ice), liquid, and gas. On the phase diagram, we can see the energy required to shift between each phase. At atmospheric pressure, water becomes gas at 100 °C and solid at 0 °C (Figure 7.8). But on this diagram, we can also observe a triple point below which the ice can directly transform into a gas. This is reached at low pressure, and it is called sublimation. This particularity of water is used to efficiently remove water using a freeze dryer. In a freeze-dryer, solid water (frozen) is sublimated under a high vacuum typically below 0.1 mbar. The water gas, steam, is collected in a condenser usually operating at –20 °C to –50 °C to trap the gas as pure water (Figure 7.8). This technique is used to create dehydrated samples and is commonly used in the food and pharmaceutical industry. The removal of water increases the shelf



life of pharmaceutical preparation. Indeed, the removal of water prevents the hydrolysis of active pharmaceutical ingredients or degradation of nutrients or vitamins in food samples.

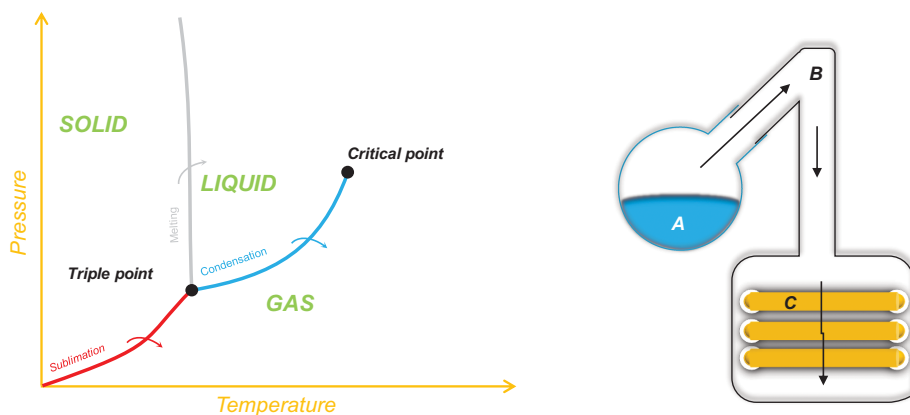


Figure 7.8: (left) Phase diagram for water and (right) scheme of the principle of a freeze-dryer utilized to dehydrate samples containing water. The (A) frozen water sample is put under (B) vacuum and the water sublimates into steam that is collected in a (C) water condenser.

7.2.5 Supercritical fluids

Polymer expansion technique uses CO_2 that can be infiltrated in the polymer matrix as a gas. CO_2 is adsorbed into the polymer matrix, and then the gas expands to create bubbles. However, the temperature at which the process occurs must be above the T_g of the polymer so as to be able to deform the matrix without inducing cracks in the polymer phase. But the addition of a gas in the polymer matrix impacts the physical properties of the polymer chains and can result in a change of T_g (Figure 7.9). Therefore, this process is highly dependent on the gas parameter and type of polymer used. Like water, carbon dioxide can be brought above its critical point under mild conditions. Above a certain temperature and pressure, the liquid and gas phases are not separated anymore, and the fluid can penetrate porous structures much like a gas. Using supercritical CO_2 permits to conduct the expansion process at a higher temperature than if it were in the gas phase. The parameter used in this process permits the creation of either closed-pore architecture or open-cell pore architecture. The technique of polymer expansion is derived from poly(styrene) foam which is a well-known consumer product for isolation materials. Applied to biomaterials, the expansion with supercritical CO_2 was adapted to PLA to create a porous biodegradable implant [12].

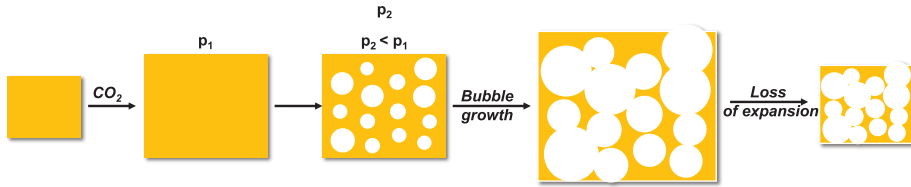


Figure 7.9: The expansion process of the polymer matrix. The polymer matrix swells upon adsorption of carbon dioxide. A decrease in the pressure lets the gas expand in the polymer matrix. Upon release to atmospheric conditions, the porous polymer matrix loses its expanded size but remains porous.

7.2.6 Block copolymer phase separation

In the late 1960s, Meier proposed using block copolymers to induce microphase separation in the polymer bulk. The microphase separation consists of a chain segregation in block copolymers in the bulk state and in concentrated solution. The structures generated by the packing of domains on the macrolattice are termed mesomorphic structures. Using Flory–Huggins interaction parameters of the polymer blends, one can predict the shape of the phase separation of the block copolymers. Microphase can be obtained depending on the ratio of each block and its miscibility to each other (Figure 7.10) [13]. If you have one block that can be leached out without affecting the other block, you can create a predictable microporous architecture. This can be used with block copolymers made of poly(butadiene) and poly(dimethylsiloxane) or PLA with poly(styrene). To reinforce each phase, one can cure or crosslink the phase that needs to be kept from the etching of the other block [14, 15].

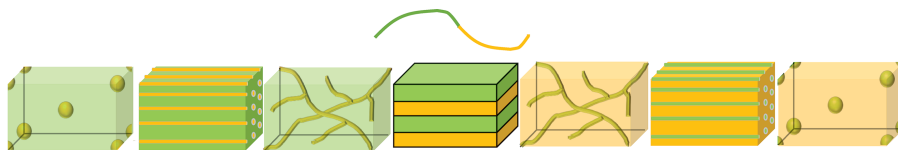


Figure 7.10: The phase separation transition observed in block copolymers can be calculated according to Meier's theory.

7.3 Microfiber-based scaffolds

If we are to reproduce the natural organization of tissues, we need to mimic the different types of natural porosity. For example, we have seen above that bone, and inorganic phases have a porous architecture that can be reproduced by using porogen. However, connective tissue, muscle, or tendon have a fibrous organization.

These tissues' organization is based on fibers made of extracellular matrix macromolecules such as collagen proteins or chondroitin sulfate polysaccharides. These polymers will then form a fibrillar linear organization.

For centuries, we have made fibers that are used for making textile. These techniques have been refined over the years to become today's textile industry. Using three main processes: wet spinning, melt spinning, and dry spinning, fibers can be produced out of biomaterials, and these fibers can be brought to use for biofabrication applications (Figure 7.11).

7.3.1 Dry spinning

In the dry spinning process, the polymer is dissolved in a solvent. The polymer solution composed of the polymer and the solvent is extruded through a spinneret. The spinneret is an extrusion nozzle through which the polymer solution passes through. The size of the nozzle will determine the size of the fiber. The polymer chains will be forced to organize into the linear trend through the fiber extrusion. After the spinneret, the polymer solution must be dry to remove the solvent. The dry or semi-dry fibers are collected into a series of wheels called a draw twister to align the polymer chains further. This drawing tends to increase the crystallinity of the polymer and increases the strength of the fibers. At the end of this process, the fiber is collected on a spool.

7.3.2 Melt spinning

It is not favorable for all polymers to be processed through a dry spinning process in a solution. Thermoplastic polymer, if they have a low melting temperature might be preferable in terms of production cost and lack of solvent to be melt spun. The process is like dry spinning, whereas in melt spinning the polymer is brought to flow by melting it and not as a solution in a solvent. This has the benefit of not using solvents that can be potential ecologically and healthy harmful. The melted polymer that goes through the spinneret is then evaporated by air, drawn, and spun on a spool.

7.3.3 Wet spinning

In some cases, polymers can only be dissolved in a low-volatile solvent such as DMF or DMSO. These solvents cannot be evaporated in a dry spinning process. But, because they are water soluble, one can extract them in an aqueous bath. The dissolved polymer is passed through a spinneret that is immersed in a water bath. The

fibers are then pulled into a draw twister for further drying of the water and then collected on a spool. The water bath can also be composed of calcium chloride and be used to crosslink alginate polysaccharides [16, 17].

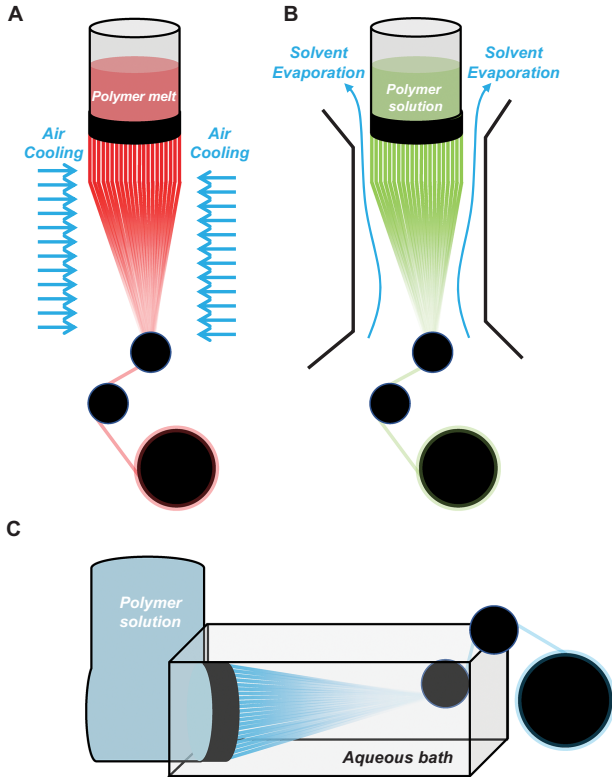


Figure 7.11: The fiber manufacturing process: (A) Melt spinning, (B) dry spinning, and (C) wet spinning.

7.3.4 Fiber bonding

Once fibers are obtained on a spinneret, they need to be bound to make a scaffold. One technique used for making micrometer scale fiber-based scaffold is to use the fiber bonding technique. Starting with individual fibers of a polymer A, the non-bonded fiber structure is immersed into a solution of a polymer B. The solvent containing the polymer B is then evaporated and we obtained a block made of polymer B with the fibers of the polymer A embedded in the polymer B matrix. Like this, the fibers made of polymer A are fixed. We can then apply a heat treatment to seal the fibers of polymer A together. Then, the polymer B can be dissolved using a non-solvent of polymer A and a fiber mesh is obtained (Figure 7.12).

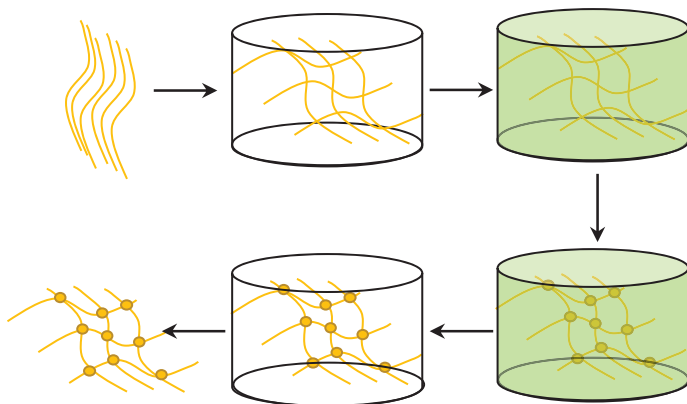


Figure 7.12: Schematic of the fiber technique bonding using a polymer matrix (green) to immobilize the fibers (yellow) and bond them through thermal treatment and subsequent removal of the green polymer.

While this technique is quite old, its efficacy has been proven for large fibers diameters. It is, however, challenging to handle micrometer- and nanometer-scale fibers and to precisely control their organization. Therefore, other techniques have been developed with the aim to gain control over the organization of the fiber in the scaffold.

7.3.5 Hollow fibers

One of the most sought-after architecture in biofabrication is hollow channels. Indeed, many tissues are composed of hollow channels such as blood vessels or lymphatic channels. Therefore, if we want to create functional tissues that can survive post-implantation in the patient, these tissues need to be connected to the host vasculature and lymphatic systems. As such, making hollow fibers has become an interesting way of engineering channels that could mimic the geometry of blood vessels. To make hollow fibers, a coaxial extrusion technique was developed using alginate hydrogel. Alginate forms a hydrogel upon crosslinking with calcium chloride. In a coaxial nozzle, a solution of calcium chloride is extruded in the middle of the alginate hydrogel precursor solution. The calcium chloride solution will induce crosslinking of the alginate and leave a hollow channel. The fiber is then extruded in a bath of calcium chloride to gel the outer shell of the fiber (Figure 7.13). Combined with a 3D bioprinter, these hollow fibers can be processed into 3D scaffolds with aligned hollow fibers that mimic the fibrillar organization observed in many tissues. Furthermore, the hollow architecture provides a channel to infuse endothelial cells that could be used to make blood vessel-like architecture [18–20].

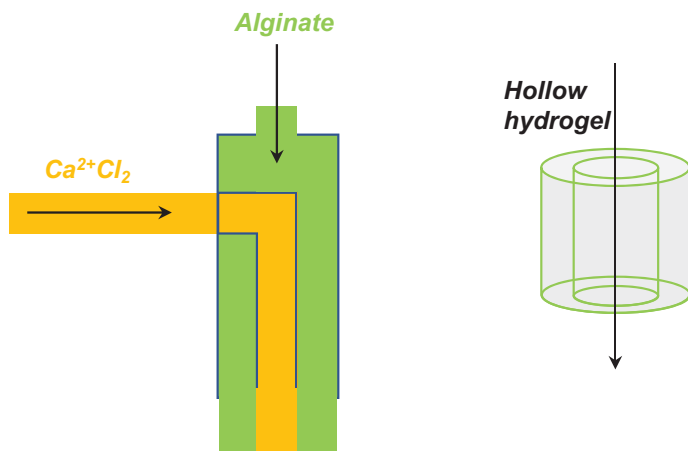


Figure 7.13: Schematic of (A) the coaxial extrusion setup and (B) resulting in hollow hydrogel fibers obtained with this setup.

7.4 Nanofibers fabrication

While these techniques allow making fibers with a diameter at the micrometer scale, the fiber diameter is limited by the extrusion nozzle diameter. To overcome this limitation a technique that could generate fibers that are smaller than the extrusion nozzle is needed. Therefore, the electrospinning process has been developed to overcome the limitation of the conventional fiber fabrication techniques.

7.4.1 Electrospinning

The electrospinning technique uses a high-voltage low-current electrical force to draw fibers out of an extrusion nozzle. The electric force permits elongating the fibers and getting fibers that are smaller than the diameter of the extrusion nozzle and obtaining polymer fibers at the nanometer scale. This technique is the result of centuries of observations and scientific advances. In the seventeenth century, it was observed that a water droplet can be deformed by an electric current generated by a rubber amber through electrostatic interaction. This observation shows that you can elongate a spherical droplet into a conical droplet. Later in the nineteenth century, Plateau and Rayleigh studied the formation of the water droplet. By letting water through a faucet, Plateau found experimentally that a vertically falling stream of water will break up into drops. Rayleigh showed theoretically that falling water with a circular cross-section should break up into drops. This is due to tiny perturbations in the stream. These perturbations exist initially in minuscule amplitudes, as time progresses, the perturbations

will grow and pinches the stream into drops. Zeleny later studied the behavior of water under electric discharge. He observed that depending on the voltage, a droplet positioned at the opening of the meniscus was delivered differently as the electric potential increased. During the beginning of the twentieth century, following the work of Rayleigh and Zeleny, Taylor studied the behaviors of a droplet under electric current to understand storms and observed that to get jetting of the solution you need to be in a particular condition where a cone is generated. Variation of the potential will disrupt the cone and will give either droplet or multijetting. In parallel to the development of the industrialization of the textile industry, small fibers that could reproduce the silk's fibers properties were sought after. The electrospinning of cellulose acetate solution gave nanometer-scale fibers that could be used as a filter for gas masks. It is now an industrial process that can produce nanometer fibers at the ton scale.

The current electrospinning setup works using a loaded syringe. The solution of polymer is extruded at a constant flow rate using a syringe pump. At the tip of the syringe, a blunted needle is positioned, and a high potential is applied between the needle and the target. As the polymer solution reaches the needle, a Taylor's cone is formed, and the polymer solution jets onto the target. As the polymer solution travel, the solvent is evaporated, and the polymer reaches the target. Once onto the target, the polymer is further dried to remove the remaining solvent. The polymer can be jetted out of the needle and is not affected by gravity. Therefore, the setup of the solution electrospinning is usually horizontal [21] (Figure 7.14).

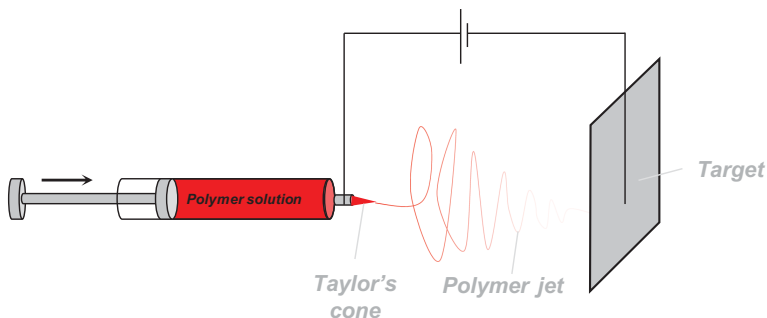


Figure 7.14: Scheme of the electrospinning setup showing the syringe, the polymer, and the target.

Following the first demonstrations on polysaccharides, research groups demonstrated that virtually every polymer solution can be electrospun. If the polymer cannot be solubilized in a volatile solvent, then melt electrospinning can be conducted, similarly to the melt spinning described above. However, because solutions of melted polymers are highly viscous, it requires a vertical setup and two current generators: one positive on the needle and one negative on the target to be able to push the polymer melt.

7.4.2 Advanced electrospinning

The extensive study of the electrospinning process has let scientists and engineers develop more advanced processes. The use of thermoplastic polymer has enabled to remove solvent and thus streamlined the process. Complex polymer solutions such as emulsion have been electrospun, and this enables the creation of complex fibers made of two polymers organized into a core-shell structure. Coaxial electrospinning like coaxial wet spinning now lets us make fibers with nanometer diameter with a hollow core to be fabricated.

But still, the major limitation of electrospinning is the lack of control over the polymer fibers' organization. It is not possible to precisely organize the polymer fibers onto the target using conventional setups as only non-wave fibers are obtained. Different setups have been proposed to try to organize the fibers. Changing the flat smooth target with a metal grid lets the fiber concentrate on the grid. Other systems used a cylinder as the collector plate. This setup gives the possibility to align the fibers along the rotation axis of the rotating collector (Figure 7.15). Combining the rotating collector with a grid setup permits to get grooves in the obtained tube. But still, a predictable deposition of the fiber and controlling the position of the fibers on the target needs to be achieved [22].

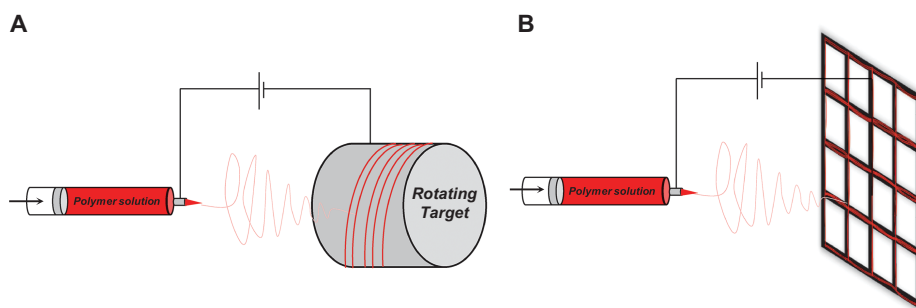


Figure 7.15: Electrospinning attempts at controlling the fiber organization: **(A)** on a rotating collector and **(B)** Electrospinning on a metal grid.

7.4.3 Melt electrowriting

The advances made in electrospinning-based fabrication processes and notably the use of polymer melt drastically streamlined the process. The lack of solvent made the technique simpler to apply. With the development of 3D printing technologies, extrusion setups that can be moved along three axes during extrusion have become widely accessible. Combining 3D melt printing with electrospinning in a process called melt electrowetting could help get a precise fiber organization. Conversely to

extrusion-based 3D printing in electrospinning, the extrusion nozzle needs to be at a certain distance from the collecting plate to avoid an electric shortcut of the setup. When extruding a viscous liquid during movement, a drag of the fluid can be observed. Depending on the displacement speed of the extrusion head, further disruption can be observed, and the extruded viscous liquid can start to oscillate. Therefore, to get a precise deposition, in addition to the right electrospinning parameter (distance from the target, extrusion speed, and voltage) the optimal displacement speed needs to be found. This speed is called critical translational speed (CTS). Below the CTS, the polymer melt is buckling, and only above the CTS, a linear deposition can be observed (Figure 7.16). This phenomenon can be reproduced at home. If you deposit ketchup on a hot dog bun, you will squeeze the viscous ketchup while moving your bottle above the bun. Only if you move the ketchup bottle at a speed above the CTS, you will get a linear deposition below the ketchup will buckle and the deposition will not be linear. [23–25]

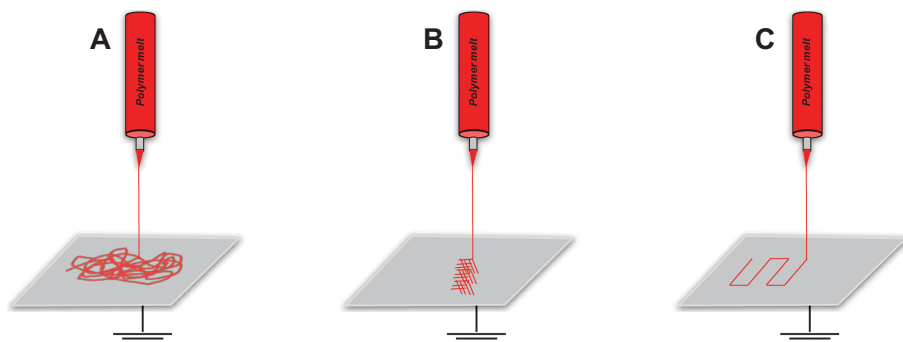


Figure 7.16: (A) Electrospinning deposition compared to (B) electrostatic writing below CTS and (C) above CTS.

The deposition of thermoplastic polymer with melt electrowetting permits to get high precision of the deposition of nanometer-scale fibers. Further development of this technique allows the user to choose the fiber size and deposit several layers of the fibers.

7.5 Cell organization on and in porous scaffolds

We have seen different techniques to create a porous scaffold. We have started this chapter by drawing the link between natural tissues that exhibit porosity and the need to reproduce this architecture to mimic this tissue *in vitro*. We have been able to fabricate porous scaffolds for several decades now, and a body of knowledge has been created by studying the interaction of these porous scaffolds with different types of cells.

7.5.1 Porous scaffold and the immune response

While presenting the different techniques to create a porous scaffold, one recurring criterion to assess the quality of the method was the ability or ease to control the pore size with a given technique. Why is this so important to be able to control the pore size? It is because the size of the pores in an implant can modulate the immune response. Like in *in-vitro* culture, an implanted material will interact with its surrounding environment once implanted in tissue. Proteins will start to adsorb on its surface and fold in a specific manner. The way the proteins fold and which type of protein can fold on the implanted materials will trigger a macrophage attack on the implant. The macrophage will then decide, depending on the type of protein they encounter, if they need to recruit fibroblast to create a collagen capsule around the implant to isolate this foreign host from the rest of the system. The material used to make the implant can modulate the immune response. But not only that, the pore size can also modulate the immune response to an implant. Depending on the size of the pore size, different types of macrophage will be interacting with the porous scaffold. An implant that has no pores will in most cases trigger the formation of a collagen capsule that will isolate the implant from the rest of the body. Pores that are about 30 μm will restrict the activation of macrophages and their infiltration into the pores while permitting the infiltration of blood vessels. Bigger pores (about 160 μm), however, will allow the infiltration of blood vessels and macrophages. Once infiltrated inside the pores, an isolation capsule will be formed. Therefore, having control over the pore size permits modulating the immune response of the implant [26, 27] (Figure 7.17).

Similarly, having interconnected pores will be a critical feature of the implant. If the goal is to have a blood vessel supply at the site of implantation, one would need to have pores that allow the infiltration of a blood vessel within the whole scaffold.

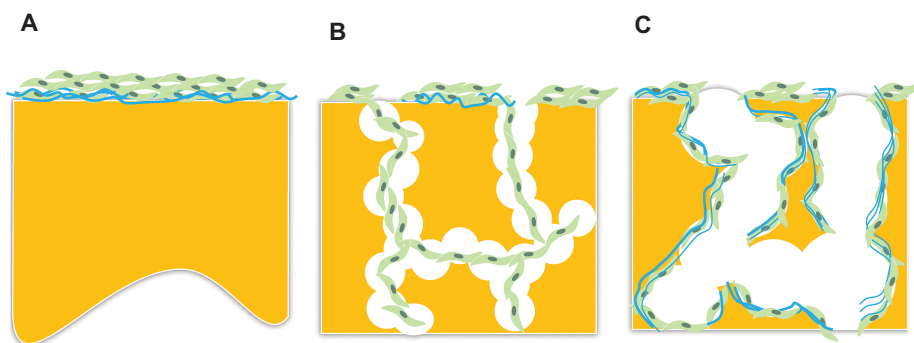


Figure 7.17: The impact of pore and pore size on cell infiltration and immune response. (A) On an implant with no pores, cells form a collagen capsule (in blue) to isolate the implant. (B) In small pores, some cells can penetrate the pore and no capsule is formed whereas (C) with an implant with broad pores a collagen capsule is generated.

For this to happen, the pores need to be interconnected throughout the entire scaffold. To have infiltration of the blood vessels, the pores need to be big enough to permit the infiltration of the blood vessel. Alternatively, the scaffold's pore needs to be degradable or elastic enough to permit the deformation of the pores for the infiltration of blood vessels [28]. Therefore, depending on the application of the implant, one must carefully design the pores' architecture and material composition. For instance, a drug-eluting implant might not require having access to the host vasculature, but a scaffold carrying functional cell such as an artificial pancreas system populated with insulin-secreting β -cell will need to have access to the host vasculature. Therefore, it is not required to have a precise pore definition for all applications.

7.5.2 The impact of fiber size

Like porogen-generated scaffolds, fiber-based scaffolds induced different cell behavior depending on the fiber size. The cell response to a fiber size can be used to define the required fiber-based scaffold for a specific application. Scaffolds with fiber diameters wider than cells are usually used to align cells in the fibers' direction. Whereas cells cultured on the scaffold with randomly organized nanofibers will tend to use the fiber as an attachment and spread between the fibers (Figure 7.18). But nanosized fiber can also be used to direct the organization of cells on the substrate. Using a rotatory target, electrospun fibers mat where all the fibers are aligned can be used to align neuron or muscle cells in one direction [17]. Within the two scales of fibers (micro- and nanofibers), subtle variation in the fiber size can also trigger different cell behavior in terms of gene expression and protein secretion. But these tremendously vary from cell type to cell type and need to be studied on a cell-type case basis.

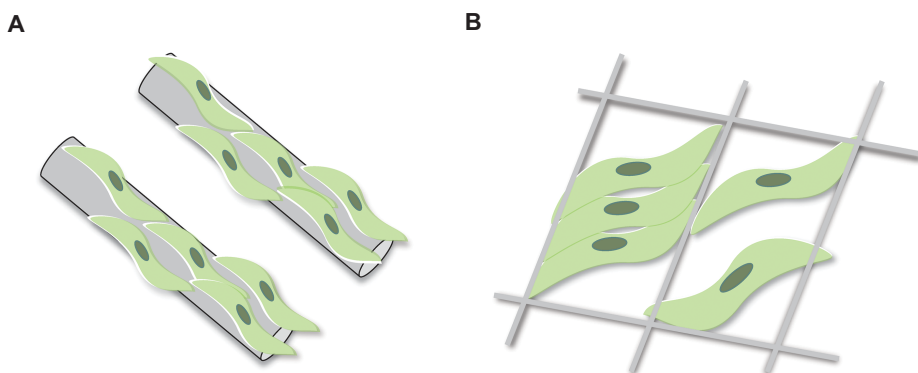


Figure 7.18: Typical organization of cells on (A) fibers with a diameter greater than the cell size (microfibers) and (B) fibers with diameters smaller than cells (nanofibers).

7.6 Quiz

1. What are the three main spinning methods?
2. Which criteria must be fulfilled to have optimal deposition in melt electrowriting?
3. What is the name of the transition of water from solid to gas?
4. What are the two types of porous networks?
5. How are polymers expanded? Example with expanded poly(styrene).
6. Which geometry is generated at the tip of the extrusion nozzle during electrospinning?
7. What type of extrusion needle is needed to fabricate hydrogel hollow fibers?
8. If I shake a recipient containing particles of different sizes, which particle size will be on top?
9. What is the definition of porosity?
10. What techniques are best suited to measure microporosity?



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8 Design strategies in biofabrication

8.1 What is design, and what is not design?

A definition of design looks at the discipline as the human power to conceive, plan, and realize products that serve human beings to accomplish any individual or collective purpose. A design is not only an object, but also a process. One can be designing a process or a way of manufacturing or achieving a task. In the more global sense, designing initiates change in a man-made thing. Therefore, the verb or the action of designing refers to the process of originating and developing a plan for a product structure, system, or component. Applied to tissue engineering, these are tissues that are man-made and not any more natural tissues. Therefore, they will undergo a design process as a human being conceives them. In developing biofabricated objects, one must have to have a plan on how to achieve this new structure. The discipline of design is at the intersection of three main fields: humanities, science, and art, as defined by Bruce Archer [1] (Figure 8.1). To evolve in the field of design, one must have scientific knowledge and know how to make an object.

Furthermore, one must have some humanities background to predict how people interact with the designed object. While design involves some artistic touch, it must not be confused with actual art. Indeed, art is made to make a statement; art makes you reflect, makes you wonder and think about something, while design works. Design is made to be functional. The difference between design and art is that design combine aesthetics and function while art is here to make you feel something, to make a statement. Going further, another difference must be clarified. It is between science and design. When sciences are concerned with how things are, design is concerned with how things are supposed to be. So, design is finding an answer to what the optimal shape of an object is. One can say that design combines aesthetics with function. Looking at two different objects with the same function, two different cars, for instance. A sports car might have a nice aesthetic but not the same purpose as a familial monospace car. Therefore, there is a different design for one object, one function, and the aesthetic of the object will affect our attitude toward the product. Aesthetic influences the pleasure we feel from the product and our long-term attitude about the product. As such, it can help us better interact with the designed object.

As an example, in the field of biofabrication, some of the 3D bioprinters currently on the market have received design awards. The bioprinter BioX manufactured by Cellink won the Red Dot Award in 2018. The bioprinter Rastrum manufactured by Inventia Life Science received the Good Design Award for the year 2020. We must now ask ourselves, what is the primary driver to design a shape for an object? The leading design theory coined by Louis Sullivan is that form follows function. That translates into the concept that the shape must be functional, and that the object's function

cannot be impaired by the form and shape of the object no matter how the aesthetic must be. If the aesthetic is the primary driver, then we leave the field of design, and the object becomes art to make a statement.

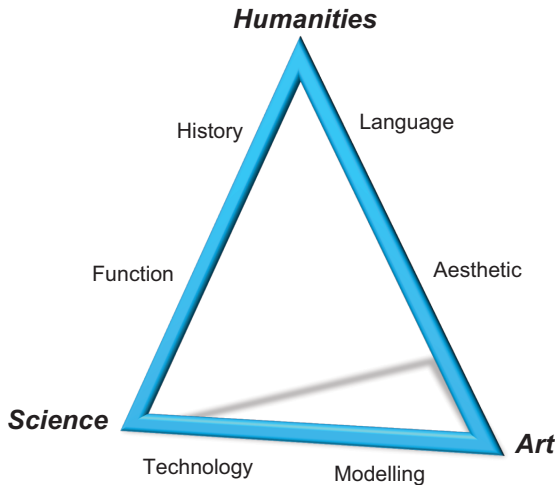


Figure 8.1: Design is at the intersection of many fields of research and performance. Combining humanities, science, and art in the design approach let designers find the best aesthetics for a functional object to predict how humans will interact with their design.

8.2 The design processes

How do we move from a design idea to an object? You will enter a discovery process following an initial idea, then build your design case, develop the idea, and test your design idea before launching your product. To assess the quality of a design, one can use different tools. One can create a matrix with selection criteria such as ease of use, performance, or aesthetics to evaluate a design. These criteria can be weighted depending on which priority or which customer your product is addressing. You will assess your design against the selected criteria using your evaluation matrix. You can give a score for each of the criteria and check if the design matches the criteria set at the beginning of the designing process. But to create this object and set of criteria, there are different approaches that will provide a conceptual framework around the design process and lead to a particular outcome. A typical design framework is called the user-centered design approach. This means that the design focuses on the end-user's needs, wants, and limitations, and these criteria will be used to define and improve the object during the design process. Therefore, you will have to identify who is the end-user and his wants and needs. In this design framework, you need to be having a great knowledge about the end-user and

then test the design against the user's needs. Alternatively, you can have an issue-based design. This design framework will solve a current problem or issue through a design. It will convert a problem or limitation into something functional and aesthetic.

Another design framework consists of using a design theory to create your object. For instance, you want to create a webpage or an advertisement. There are theories that can be leveraged to help identify the best color to convey a specific message, how to use letter alignment to facilitate the reading, or how to place an image to capture the customer's attention. In architecture, you also have design theories or design schools that have emerged across the centuries. One famous school of design was the Bauhaus movement which redefined a method to design buildings. As a designer, one needs to predict a future state based on current information. An architect needs to predict how its clients will live and move within the house he is designing based on the current situation their clients are. One must be able to predict how the design of the house might also change the way his clients are currently perceiving space versus once they will be in the new house.

8.3 The design approach in biofabrication

How is design related to biofabrication? In biofabrication, we are trying to design synthetic tissue models to obtain living objects that are here to restore a function, regenerate, or reproduce natural tissue function. One must predict how the cells will react when exposed to a scaffold, how the human body will react to the shape of implants. We need to be aware of these concerns and limitations when designing artificial tissue for *in vitro* or *in vivo* applications. Biofabrication aims at engineering living tissue using industrial manufacturing approaches. These require four steps: design, material selection, manufacturing, testing, and evaluation. As we have previously discussed, designing an object can be achieved by following different approaches: design theory, user-centered, or issue-based approach. Designing an object following each of these approaches leads to objects with different forms, shapes, and performances while having the same function. Taking a biofabrication design challenge: designing vasculature, let see what the different design frameworks are, and what the resulting outcome could be.

8.3.1 Biomimicry

Biomimicry is defined as the design and production of materials, structures, and systems that are modeled on biological entities and processes. Therefore, in a biomimicry design approach, the blood vessels would be shaped following the shape of natural tissue. In this approach, the shape of the object will be acquired from the

medical image of an adult vascular tissue [2, 3]. If we consider a blood vessel as an example, angioplasty can be used as a source of the design. The resulting image can then be processed into a computer aided design (CAD) file which can be used for manufacturing objects using biofabrication methods such as 3D printing (Figure 8.2). The synthetic reproduced adult tissue can then be compared to the original by comparing the blood flow and pressure. This is currently one of the main approaches followed to engineer synthetic tissue and artificial organs. However, this requires the precise organization of single cells into a hydrogel to form a three-dimensional structure, leading to an engineering race toward precision and speed of cell deposition. This approach is based on the hypothesis that the complexity of the mature functional tissue can be reproduced by copying the observed final architecture. But this reverse engineering approach omits the stepwise developmental processes that have led to the mature functional tissue. In an anthropomorphic analogy, we could say that tissues created by copying the final outcome lack the necessary experience for becoming mature.

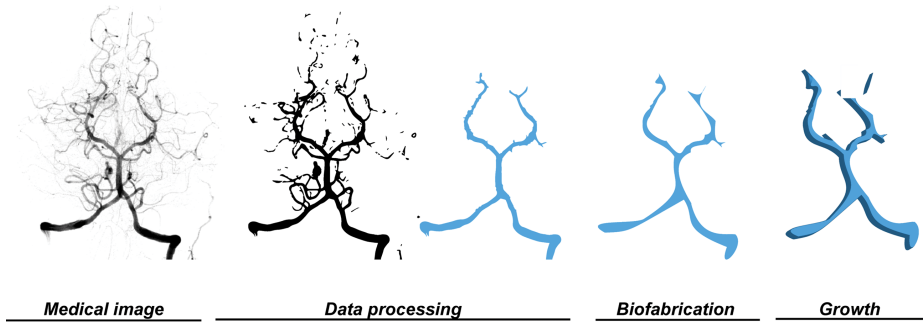


Figure 8.2: Conceptual steps followed to create tissue models with a biomimicry approach. This consists in producing natural tissue from a medical image processed into a file that can be used for digital manufacturing process.

8.3.2 User-centered

Conversely, in a user-centered approach, one tries to answer the needs of the user. Therefore, one must understand the needs and limitations of the user. If we want to design a blood vessel network to supply oxygen and nutrient to pancreatic islets, then the pancreatic islets spheroids could be considered as a user. The amount of nutrients and oxygen required by the cells can be obtained from physiological studies, and pancreatic islets must be in a 150 μm vicinity of a blood vessel to not undergo hypoxia (Figure 8.3). We can generate a design where the spheroids are placed in a tissue with a blood vessel network designed to pass no further than 150 μm from each pancreatic islet. Using this approach, a design of blood vessel is obtained that answers to the needs of the user, here the pancreatic islets. [4]

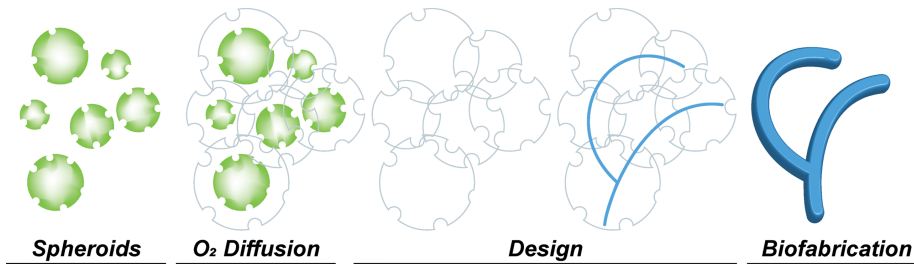


Figure 8.3: Conceptual steps followed to create tissue models with a user-centered approach, where the pancreatic islet (in green) is the user needing oxygen supply from blood vessels that are not further than 150 μm .

8.3.3 Design theory

Alternatively, a theory-based approach can leverage seminal tissue engineering results. In the case of designing a blood vessel network, it has been shown that the pore size of a scaffold can be used to control the infiltration of blood vessels [10]. Using this principle, one could design a scaffold with various pore sizes to control the shape of a blood vessel infiltrating a scaffold. This approach could also be done *in silico*, by creating an artificial porous matrix in a CAD software and letting a blood vessel find the most efficient path in the virtual matrix. The blood vessel design can then be extracted, processed, and bioprinted [5] (Figure 8.4).

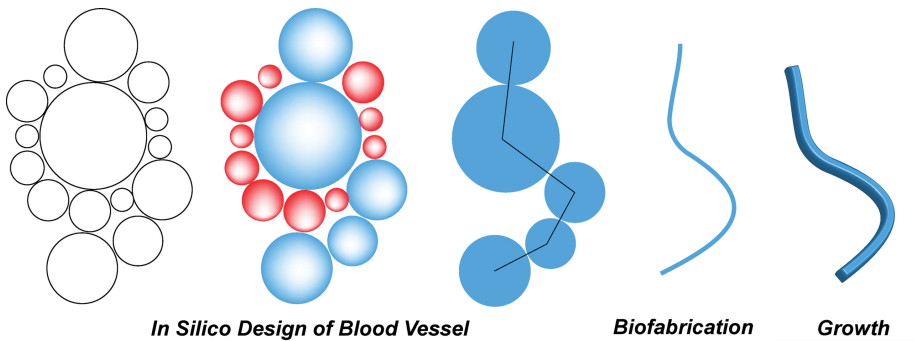


Figure 8.4: Conceptual steps followed to create tissue models with a design theory approach using *in-silico* design of a blood vessel, where a porous scaffold is used to guide the growth and path of a blood vessel.

8.3.4 Morphogenic approach

Incidentally with the establishment of robust gene sequencing instrumentation, most attention has been brought to the role of gene expression in regulating the self-organization of cells into functional organs, so-called morphogenesis. While an open-loop view where genes alone control morphogenesis has been proposed, increasing evidence for a closed-loop is collected where genes determine cell properties to execute morphogenesis based on the physicochemical properties of their environment, inducing new interactions and regulation of genes. In this closed-loop model, materials, which through physical and chemical interactions with cells, could be used to influence cell motility. During development and in a cancerous tumor, cells will undergo the epithelial-mesenchymal transition through which they will become motile. Once capable of movement, cells will move following a gradient of soluble signals, called *chemotaxis*; gradient of mechanical properties, *mechanotaxis*; and gradient of immobilized signal, named *haptotaxis*. Reproducing guided signals that induce a coordinated cell movement like what is observed during morphogenesis would allow for guiding cells through a natural developmental phase toward their final engineered adult functional tissue. Therefore, an alternative to mimicking the final organization of the blood vessel network like in the biomimicry approach and avoiding the necessity of having the technological advances to organize single cells rapidly and precisely would be to design a cellular environment that directs the self-organization of single cells into such mature tissue. In this way, one would have to create signaling patterns that will direct cell assemblies toward their self-organization into blood vessels. One could draw in a synthetic growth environment such as an hydrogel, a signaling gradient that will guide endothelial cells to organize into a blood vessel network (Figure 8.5). [8]

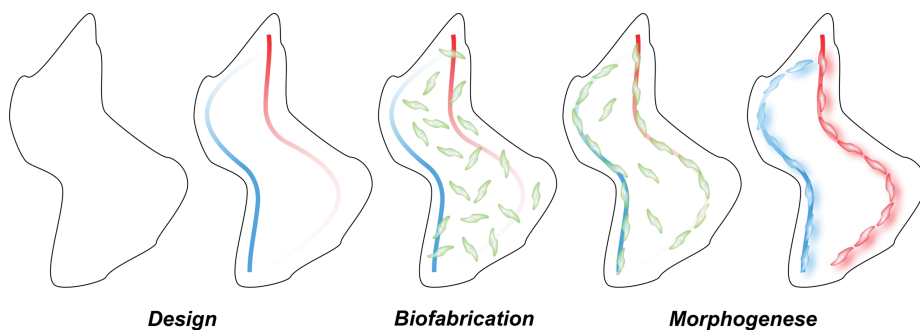


Figure 8.5: Conceptual steps followed to create tissue models with a morphogenic approach. Here, a gradient of signals is coded into the cell culture milieu that will guide single cells to organize into a blood vessel network.

8.4 The tools for designing objects

Before the computer era, the engineering and designing of an object as complex as an airplane was made on paper, and each engineer was designing the parts by drawing them on paper in 2D following a strict nomenclature. This approach was manpower intensive and cost a lot of time. Then came the first computer-aided (CAD) design software which comprises a set of methods and tools to assist the product designers in creating a geometrical representation of the artifacts they are designing. This software can help with the dimensioning and tolerancing of the parts and permits the exchange of parts and part assemblies between teams and organizations, for instance, between conception and manufacturing or between conception and maintenance.

8.4.1 Computer-assisted design

Today, one can have a whole airplane with all its parts represented on a computer program. But to arrive at this stage, many improvements were made over the past decades. At the beginning, engineers were trained to draw on paper, and so the first CAD software was developed to be used with an interactive pen. Over the years, alongside the gain in computer power, programs have become more complex, and now it is also possible to conduct mechanical and aerodynamic tests on a virtual parts in a computer program. Initially, the development of the CAD programs was mainly driven by aviation companies such as Dassault and Boeing that wanted to improve the manufacturing process. These programs are based on vectorial drawing. So, there is a major difference between CAD programs and your common drawing software. Indeed, when

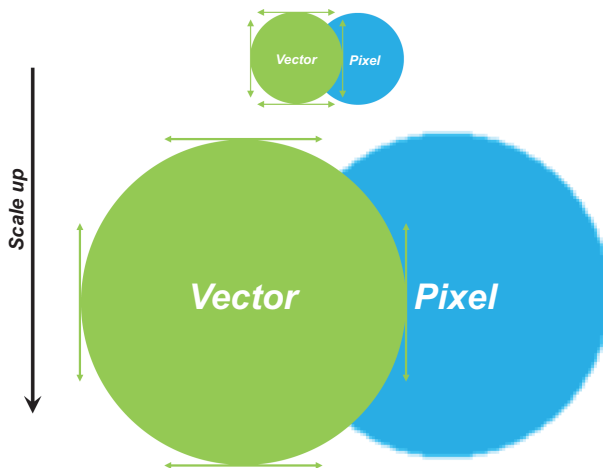


Figure 8.6: The difference in picture quality upon scaling up a vectorial drawing versus a pixel drawing. While the vector drawing does not lose resolution upon resizing, the pixel image becomes blurry.

we see a picture on a screen, we are looking at a map of pixels lighted by a diode of a different color. If we scale up this object, we will reach a limitation: the resolution of the screen used to display the object and so the resolution of the digital file will be visible, and soon the square pixels will be visible on the screen (Figure 8.6). Conversely, if we represent our object as a vector when we scale up the object, the computer has all the information to recalculate a bigger object using the given vectors. By doing so, we can scale up the object without losing resolution. The other advantage of vector drawing is that the designer can zoom in and out of the virtual object on its screen and rotates the object to be able to inspect it from all angles. Something that was not possible previously when the object was drawn on paper.

8.4.2 Industrial drawing approach

Because all CAD programs represent objects as a vector, this necessitates a different drawing approach. The operator will not directly draw a line or a 3D object such as a cube. Instead, one must start with a point. Adding a direction will allow drawing a line. We have now an object with one dimension. One must add another vector (point, direction, and length) to add another dimension. We obtain a square plan from a line, and we have an object with two dimensions. The third dimension is obtained by adding another vector to obtain a cube (Figure 8.7).



Figure 8.7: In vector design approach, a point and a direction gives a line (1D), then adding a second vector gives a plan (2D) adding another vector through extrusion gives a volume (3D) arrow represent the vector.

Beyond the resolution, vector drawing has the advantage of managing mathematical objects (vectors) to which we can apply a mathematical formula to modify the object. The drawing process can incorporate Boolean operation to subtract, add, or combine objects. For instance, to create a cube with a cylindrical hole (Figure 8.8). One can create a cube and a cylinder and subtract the cylinder from the cube. Finally, the obtained objects that are mathematical objects can be used to make mechanical calculations such as strength or aerodynamic calculation. This allows optimizing the object before the manufacturing of a prototype. To date, many programs are available to design objects. Some are available online free of charge and can be used to create a simple shape that can then be used to create a scaffold, 3D printed objects, or even bioprinted objects. There are also many online libraries to exchange medical images, which can then be used for 3D bioprinting following a biomimicry approach.

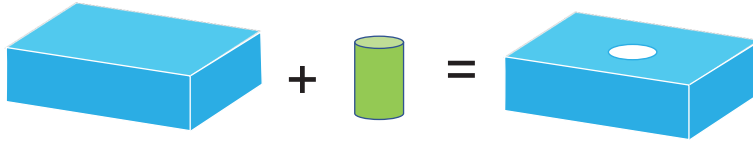


Figure 8.8: In CAD software, Boolean operations can be used to make complex objects through addition or subtraction of volumes.

8.4.3 Parametric design approach

Designs on a computer are expressed and treated as mathematical objects. We can create a parametric equation that is defined by a set of functions that expresses each variable of the equation as a function of another variable. This is particularly used for designing a building to take into consideration sun reflection and in designing mechanical parts to reduce the weight without affecting the mechanical resistance of the part. For example, designing a porous scaffold can be created *in silico* by providing the computer with a set of parameters as designing rules. As an example here, we designed a porous scaffold *in silico* using a parametric design software. Compacted beads have a set of points at their intersection that can be linked into a wireframe. The wireframe is then transformed into a volume giving a porous scaffold. Asking the computer to rearrange the beads in a random manner will give a different porous scaffold. Such scaffold is created without the intervention of the designer apart from the initial set of parameters. (Figure 8.9).

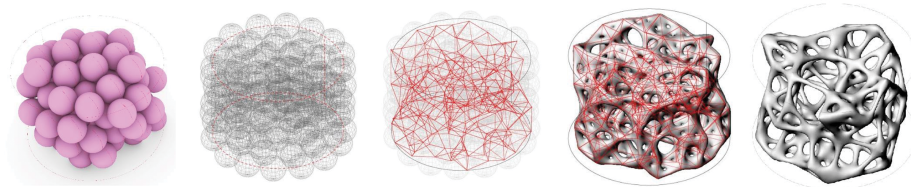


Figure 8.9: Porous scaffold created by parametric design. Beads are packed, and the intersection of these beads form a dense wireframe that is converted into a volume create a porous scaffold [6].

With all the tools available to create designs, today designers have many choices to choose from when they want to create an object. In this chapter, several ideas have been proposed on how to begin a design reflection. It was highlighted that the starting point is critical in dictating what the object will look like. Always driven by the function, a design can have different aspects depending on the point of view the designer begins with, driven by the end-user, solving a problem, or following a theory. It is the responsibility of the biofabricator to ask himself the right questions and test his hypothesis using evaluation criteria to obtain the best design for its functional synthetic tissue.



8.A Chemotaxis

Chemotaxis is the movement of cells following a gradient of concentration of soluble molecules (Figure 8.10). During embryonic development, one of the most studied examples of spatially growth factors is the zebrafish lateral line development guided by a gradient of stromal cell-derived factor 1 (SDF1) chemokine. In adult tissue, gradients of growth factors have been shown to guide the sprouting of new capillaries. This process is directed by a gradient of vascular endothelial growth factor A (VEGFA) that will be followed by tip cells leading the growth of the new blood vessel. [7]

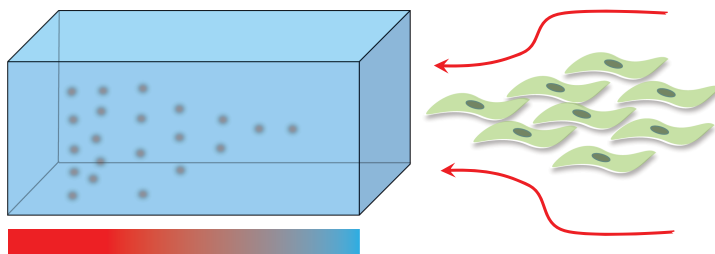


Figure 8.10: Chemotaxis: cells attracted by an increasing concentration of soluble molecules. These molecules are either trapped in a reservoir of the extracellular matrix or generated by a different cell population.



8.B Mechanotaxis

Independent of the presence of gradients of soluble signals, studies on embryonic and adult tissues have revealed the role of anisotropic mechanical properties in directing cell motility, the so-called mechanotaxis. Within this class of phenomena, it is to distinguish between *ten-siotaxis* and *durotaxis* consisting in substrate tension migration and substrate stiffness migration, respectively [8, 9].

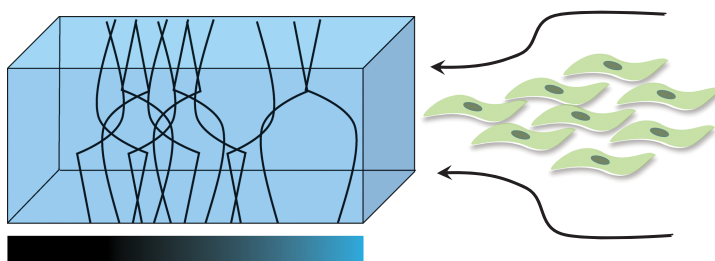


Figure 8.11: Mechanotaxis: cell movement guided by a gradient of mechanical properties generated by a gradient of material or crosslinker density.

8.C Haptotaxis

Haptotaxis is the directional motility of cells following a gradient of cellular adhesion. In this form of cell guidance, cell movement is directed by a gradient of immobilized signals on the extracellular matrix. During embryonic development, the ECM is changing to guide and support morphogenesis. For instance, in the avian embryo, it was discovered through texture analysis that the fibronectin network is organized in an anisotropic manner and that this organization is evolving through the different developmental stages [10, 11]. Thus creating a gradient of cell adhesion signalling.

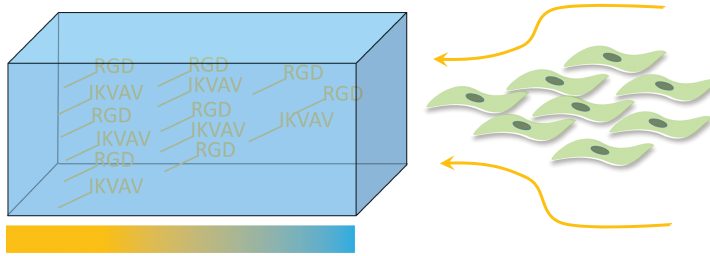


Figure 8.12: Haptotaxis: cell movement guided by the anisotropic organization of immobilized signals on the extracellular matrix, here represented by the cell adhesion peptides RGD and IKVAV.

8.5 Quiz

1. What is design?
2. What is the difference between design and art?
3. What is the relationship between function and form?
4. Which discipline intersects to fuel the design discipline?
5. Gives four different design approaches.
6. In biofabrication, what is the biomimicry approach?
7. What principles of developmental biology are used to create a morphogenetic design approach?
8. What is CAD software?
9. What is the difference between a vectorial and a pixel image?
10. What mathematical operator can we use in CAD to create an object?

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9 Bioprinting

9.1 Manufacturing

The usual techniques used for manufacturing parts consist in removing materials; for instance, machining parts using computer numerical control (CNC) machines. In this process, you start with a metal or woodblock, and you will manufacture the object by removing the metal, or wood (Figure 9.1). The second highly used industrial manufacturing method is molding. It is brought to use for metal and plastic. In the plastic industry, melt extrusion is one of the favorite manufacturing techniques. Pellets of thermoplastic are heated up in an extruder. The melted plastic is pushed through a die by an infinite spin screw, and then the plastic is molded or extruded as a filament.

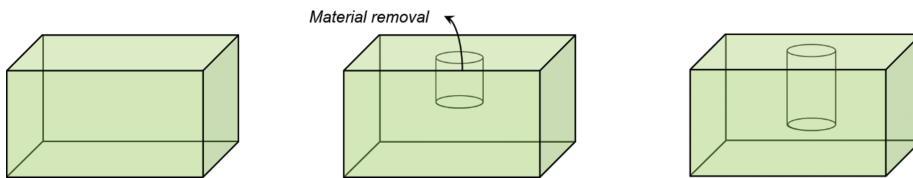


Figure 9.1: Material removal approach starting with a block, the materials that are not needed is removed and wasted.

Conversely, additive manufacturing (AM) does not rely on removing materials but on adding the material needed to create the part. Only the material needed to make the part is utilised. In terms of materials use it is similar to the extrusion molding approach. Indeed, materials such as a thermoplastic is added layer after layer to make the final desired object (Figure 9.2). However, AM parts need to be designed with a different concept and approach. removing versus adding materials makes a significant difference. Some objects can only be manufactured with a material removal approach and others only with the AM approach. In general, the AM techniques permit to manufacture more complex parts than the machining and molding approach.

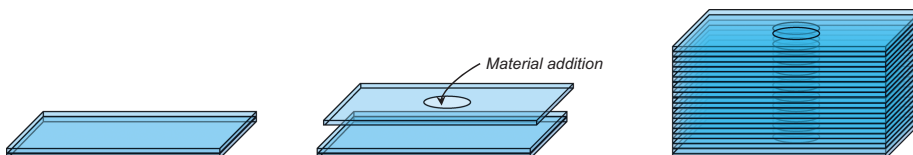


Figure 9.2: Additive manufacturing approach where only the needed material is utilized to make the part.

9.2 3D printing

In early 1984, 3D bioprinting emerged with the first patent from Charles Hull, who then launched 3D Systems with the stereolithography (SLA) process. Two years later, the first patent for the fuse deposition modeling process consisted of a thermoplastic extruder placed on an x,y,z -axis. The SLA process works with a resin that is UV activated for crosslinking, giving a thermoset material, while fuse deposition modelling (FDM) is reserved for thermoplastic. In early 1990, the first industrial 3D printer was released. At the beginning of the technique, 3D printing was mainly reserved for industrial processing and reserved for prototyping. As the method developed, the cost was optimized, and processes fastened, it slowly became a possible means for manufacturing functional parts. In 2009, as the initial patents on 3D printing expired, MakerBot proposed a 3D printer for the consumer market. Made of assembled wooden panels, the MakerBot 3D printer brought 3D printing into the limelight and democratized this technique worldwide. Now, everyone has access to this technology and can play around and develop an application. Each consumer now has the potential to manufacture parts at home. Ever since the technique has improved and materials quality and range increased, the industry has flourished. Indeed, 3D printing technology promises to reorganize the whole manufacturing and supply chain industry by relocating the manufacturing closer to the end-user or even at the end-user site. Thus, reducing the manufacturing time, shipping, and reliance on external manufacturing suppliers. With the now availability of cost-effective 3D printers, new applications in the biomedical field emerged. Over the past decades, different manufacturing 3D printing techniques have been brought to the market. Each with their advantage and drawbacks, which ultimately gave the digital manufacturing world a plethora of techniques to play with.

9.2.1 Stereolithography

A resin that can be crosslinked by UV light is brought into a bath that can move along the z -axis (height). Then, a laser is shined into the matrix to crosslink the material. Only the resin lightened by the laser is crosslinked. The part of the resin not in the laser path remain liquid and uncrosslinked. The resolution of the object is determined by the precision of the laser and the precision of the crosslinking chemistry of the resin. Indeed, radicals generated by the laser can travel into the resin and polymerize materials outside of the laser path. But by carefully choosing with the SLA technique crosslinker activator, one can obtain high-resolution parts. The part received is a thermoset object which cannot be melted anymore and cannot be reused.

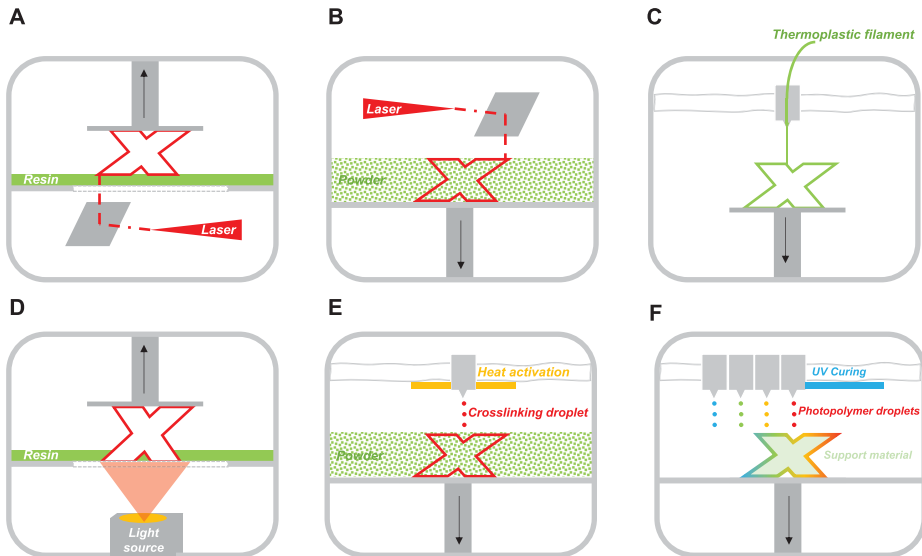


Figure 9.3: The three main additive manufacturing techniques: (A) stereolithography (SLA), (B) selective laser sintering (SLS), (C) fused deposition modeling (FDM), (D) digital light processing (DLP), (E) multijet fusion (MJF), and (F) polyjet technology.

9.2.2 Selective laser sintering

The sintering process consists of compacting materials by heat without melting. A powder of polymer materials (or metal) is placed on a stage in the selective laser sintering (SLS) printer. A laser will then heat the surface of the powder to sinter (compact the powder) to form a solid object. Because the material does not have to be melted to be sintered, high melting temperature materials can be processed by this technique. For polymer materials, polyamide and PEEK are usually processed by SLS due to their semi-crystalline properties and high melting point (268 °C and 343 °C). During this process, the heat permits the polymer molecules in the powder particle to travel from one particle to another one, thus fusing the particles together.

9.2.3 Fused deposition modeling

Using thermoplastic materials, fused deposition modeling (FDM) enables to create plastic parts from biodegradable polymers such as PLA or PCL. First, the material is provided as a filament rolled on a spool. Then, the thermoplastic polymer filament is guided into an extruder that heats the filament to melt the polymer. The melted polymer is pushed out of the extrusion nozzle (die) by the incoming filament continuously feeding the

extruder. In this technique, the nozzle diameter is the limiting factor for the resolution. In contrast to the laser-activated 3D printing technique, the resolution achieved with fused deposition modeling (FDM) is lower due to the mechanical limitation of the extrusion nozzle. The smaller the nozzle is, the more force is required to push the materials out of it. Therefore, this technique is usually reserved for a polymer with a low melting temperature.

9.2.4 Digital light processing

Digital light processing (DLP) is similar to SLA as it is using a photopolymer that crosslink into a resin upon light activation. But instead of using a laser to trace a layer, digital light processing (DLP) uses a projected image that allows cross-linking an entire layer at once. The technique is faster than SLA and is used to create intricate designs, but the size of the printed object is limited by the light source and thus reserved for small objects. In addition, because it uses light instead of a laser, the resolution of the parts is often lower. However, the latest improvement of the light source quality and resolution has permitted to increase the quality of the DLP-produced parts.

9.2.5 Multijet fusion

Usually reserved for nylon 12, multijet fusion uses a bed containing the polymer powder. Then, a liquid crosslinking agents are added; these do not react directly. The powder and the crosslinking agents are then heated, fusing the activated nylon particles. Once the build is cooled, the unreacted powder can be removed and reused for other manufacturing. The printed parts are then post-processed using water or air blasting to remove unreacted polymer powder. One of the limitations of this technology is the grey colour of the obtained objects and the few materials range available, although now different types of polyamide and polyurethane are becoming available which is expected to extend the range of applications for this technique.

9.2.6 Polyjet

Parts that need to be colored are usually printed with polyjet technology. This method uses droplets of liquid polymer, which can be colored. The polymer droplets are deposited on a bedplate and are directly cured by UV once they are deposited on the bed. Because it uses several inkjet printer heads, parts can be printed in a broad range of colors and materials properties.

9.3 3D printing for medical applications

It has always been of high interest for surgeons to obtain information on their patient's anatomy and condition before surgery. Typically, this is achieved by medical imaging obtained by X-ray computed tomography or magnetic resonance imaging (MRI) which directly give a digital 3D representation of the internal organs. Although virtual reality glasses have permitted to help the surgeon to have a better feeling about the organs in 3D, nothing can beat a real object. This is how 3D printing made its way to the hospital by giving the possibility to reproducing an organ outside the body. In preparation for complex surgeries, the surgeon can transfer the digital images obtained by CT or MRI into a 3D printed plastic replicate of the organ made using one of the techniques described above (Figure 9.4). Using the 3D printed model permits visualization and drawing a strategy for the surgery before getting to the patient. Some of these digital images obtained by CT or MRI into plastic models can be used for training purposes as well. The doctor can rehearse the required movement before the operation using the exact model of the patient's pathology. These replicated plastic organs have also been helpful for doctors to explain complex pathologies to a patient and to reduce anxiety by showing and explaining the planned procedure.

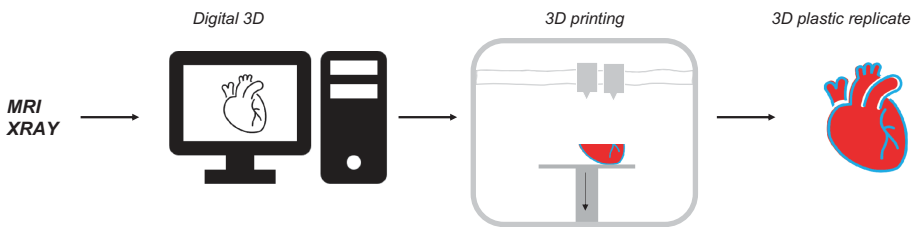


Figure 9.4: Workflow of 3D printing of plastic replicated organs for educational and surgery preparation purposes.

In parallel to this educational and training application of 3D printing, the potential of 3D printing became apparent for the manufacturing of patient-specific implants. For example, patients suffering from bone injuries often require a metal implant, which needs to be custom-made to fit their anatomy and pathology. Highly skilled and precise mechanics can machine such implants. The introduction of digital manufacturing enables to optimize the process of designing the implant from imaging file (MRI, X-ray) into a manufactured part that can directly be fitted to the patient.

9.3.1 Metal printing

Typical bone implants are manufactured out of metal alloys. With the emergence of metal 3D printing using laser and electron beam sintering, it is now possible to create patient-specific metallic implants with these technologies. Following a typical digital manufacturing workflow, metal implants can be custom-made for patients.

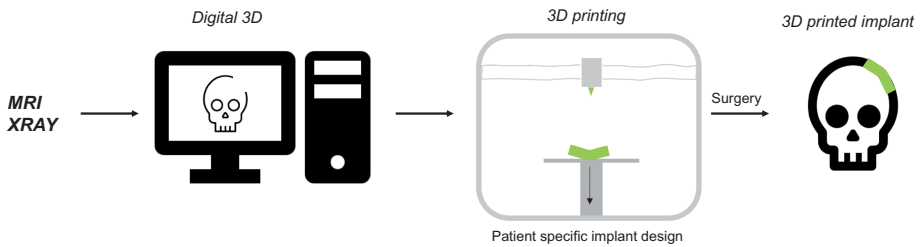


Figure 9.5: Workflow for the digital manufacturing of patient-specific implants. Anatomical information is obtained from medical imaging. Next, the implant is designed *in silico*, 3D printed, and then implanted.

9.3.2 Plastic printing

Two materials are currently used for plastic-made implants: polymer of the PEEK family and PCL. While PEEK polymers are extremely tough and can replace metal implants in some applications, they are challenging to process because of their high melting temperature. Conversely, PCL has a low melting temperature and is biodegradable. Therefore, PCL is mainly used for applications where osteoregeneration is needed, meaning the implant degrades as the bone fracture heals. The ongoing development of PCL implants aims to generate drug-eluting implants to support tissue regeneration. Due to their low melting temperature, it is possible to add drugs to the polymer melt without damaging the active pharmaceutical ingredient. Furthermore, by optimizing the workflow of implant manufacturing, a reduction of manufacturing cost is expected, and better integration of the scaffold with the patient anatomy.

Dentistry is the leading consumer of medical implants. Because implants are not positioned in a critical part of the body (lung, heart, liver, or kidney), and the implant is not inside the patient, it can thus be easily removed in case of failure. Also, many dental prosthetics are not kept by the patient and are removed at night. Therefore, the time in contact with the body is restricted. Because of these, the regulation for materials used in dental applications are less stringent than for other implants. In addition, the UV-activated materials that are used for 3D printing use similar chemistry than the resin used for dental repair. So, there is a long-standing relationship between dentistry and polymer implant which has led to a great experience with these

materials and an excellent knowledge of the interaction of these polymers with the body. With the aim of replacing complex dental prosthetic manufacturing, reducing the cost of manufacturing and revolutionising the prosthetic workflow manufacturing, dental 3D printing is slowly gaining market share and adopter.

9.4 3D bioprinting

Cells have been cultured on a planar substrate such as a petri dish for over a century. But this type of culture does not support cells to organize into a natural structure. For instance, endothelial cells cultured on petri dishes have a random organization, while in the liver, they organize into luminal shapes to form blood vessels. If we want to reproduce the function of natural tissues *in vitro*, then the cells must resume their biological organization *in vitro*. Indeed, endothelial cells have a principal role in forming blood vessels that carry blood. However, endothelial cells cannot achieve this function when the culture is in 2D on a petri dish. Therefore, culture models that can force endothelial cells to recapitulate this function are needed.

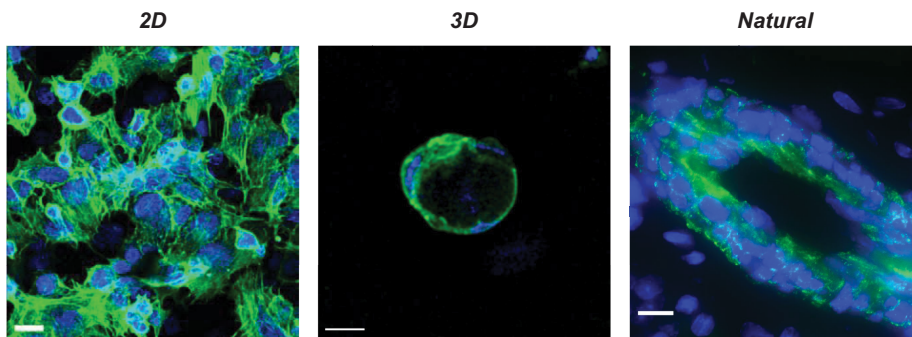


Figure 9.6: Endothelial cells cultured on a petri dish (**2D**) compared to the same cells in a hydrogel (**3D**) and a mouse kidney (**natural**). Adapted from Labor und More Issue L&M 2 / 2014.

This is where 3D cell cultures come. As tissue engineering emerged in the late 1990s rapidly, hydrogel-forming materials were used to culture cells in 3D, which provides cells with the necessary volume to resume their natural function. But as the engineered tissues evolved, it quickly reached a limitation as most of these tissues were made through tedious manual methods. When the 3D printing technologies were democratized, tissue engineers saw a potential tool to process living biological materials reproducibly and in an automated manner. Replacing bones and teeth with plastic or metal materials only replaces and mimics a small percent of the body tissue and does not include living cells. So, hydrogels used in tissue engineering

were adapted into a 3D bioprinting process where the cells and the hydrogel could be processed into a tri-dimensional object.

9.4.1 The materials of bioprinting

Leveraging the advances made on 3D cell culture, a range of bioprinting compatible hydrogel materials are now available. Pluronic, a synthetic block copolymer, is often used as reference material for testing bioprinting parameters. Because of its low critical saturation temperature, it exhibits a gel to solution transition at about 4 °C [1]. This is particularly interesting for using Pluronic as support materials that can be later removed by lowering the temperature. Other materials are extracted from animals: collagen can form a hydrogel by crosslinking the polymer chain into a triple-helix. A high concentration of collagen can be extruded and to form a support for cell proliferation [2, 3]. Gelatin modified with methacrylate groups permits to obtain an extrudable hydrogel that is then crosslinked under UV light [4]. Other hydrogels are extracted from marine seaweed: agarose [5] and alginate polysaccharides [6, 7]. Agaroses undergo a thermal solution to gel transition at about 37 °C and a gel to solution transition at 90 °C. This gives bioinks that are thermally stable. Alginate forms a gel when mixed with calcium chloride solution. Finally, nanocellulose is extracted from plants and forms a shear-thinning colloidal suspension [8]. Some hydrogel materials are used alone or in a blend, combination of different hydrogel forming materials permits to achieve the optimal mechanical properties for a specific tissue and a particular bioprinting method. Loading cells in hydrogel materials was inherited from the advances made in tissue engineering. However, not all hydrogel materials are compatible with every type of cell and every bioprinting method. Loading cells into a hydrogel in a formulation that is processable by a bioprinter was named *bioink*. The formulation has to be optimized for each bioprinting method using an established repertoire of biomaterials [9] (Table 9.1).

Table 9.1: The principal hydrogels used in 3D bioprinting.

Name	Type	Crosslinking
Pluronic	Synthetic	Physical (LCST)
Gelatin methacrylate	Natural (animal)	UV crosslinking
	<i>Chemically modified</i>	
Collagen	Animal	Physical (triple helix)
Agarose	Natural (algae)	Physical (UCST)
Alginate	Natural (algae)	Physical complex
Nanocellulose	Natural (plants)	Colloidal suspension

9.4.2 The bioprinting methods

The bioprinting technology was pioneered by modifying an inkjet printer and shown that inkjet technology could be used to print living cells in a hydrogel matrix. Now, advances made by the 3D printing industry are adapted to bioprinting, and the number of methods is constantly increasing (Figure 9.7). But processing a living material is quite challenging as the method needs to be compatible with the precious and fragile cells [10].

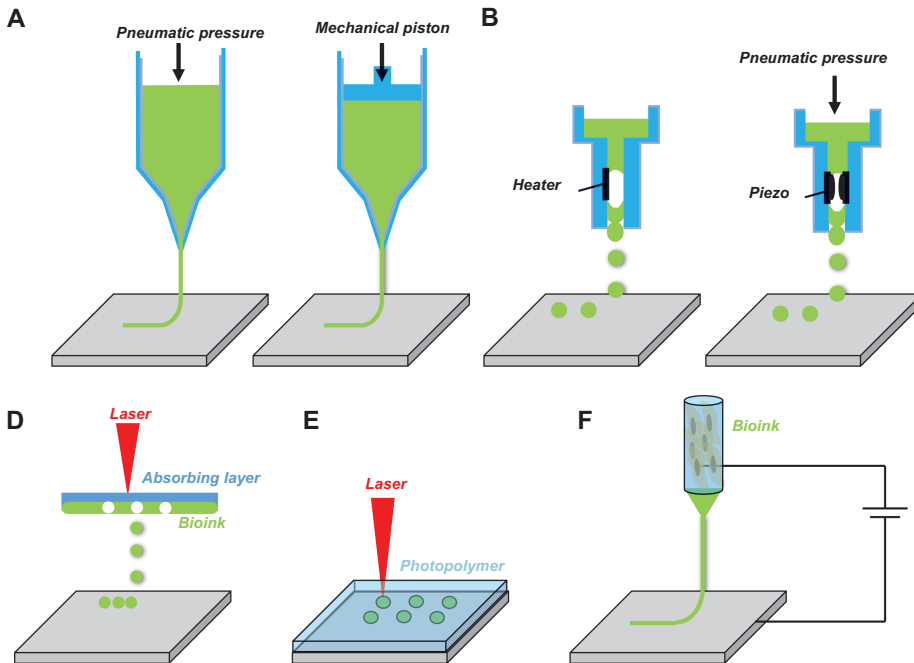


Figure 9.7: The main bioprinting methods are (A) extrusion printing, (B) inkjet printing, (D) laser-assisted droplet, (E) stereolithography, and (F) electrohydrodynamic printing.

Inkjet/drop-on-demand

The inkjet and drop-on-demand bioprinting consist of extruding materials as a droplet on a substrate. But these methods differ in the way they generate droplet and their size. Inkjet technology is directly derived from the printing industry. A droplet is extruded by generating pressure in a loaded channel. The one method to generate pressure is by heating the bioink, which dilates the materials and creates a pressure point. In this technique, the droplet is continuously ejected by the printer. Alternatively, the bioink container can be pressurized, and a piezo element or a valve

controls the opening of the cartridge, providing control over the deposition of the droplet, giving a drop-on-demand process [11–13].

Extrusion

With the democratization of fuse deposition modeling 3D printers, now access to reliable, cost-effective extrusion platforms was granted. It was then possible to change the filament extruder of a commercially available 3D FDM printer by a syringe pump to push a bioink out of a cartridge. The extrusion process relies on a syringe loaded with the bioink (hydrogel and cell) that is pushed out of the nozzle through pneumatic pressure or by a mechanical piston. While both techniques are currently available, mechanical extrusion offers several advantages such as precision as a motor rotates a screw that pushes the piston. The precision is given by the thread of the screw and the accuracy of the motor. In pneumatic extrusion the materials is pushed by compressed air, which is more difficult to precisely control with low cost equipments.

Laser-assisted

Using the same principle of to inkjet extrusion (using heat to generate a bubble), an alternative to inkjet printing was developed using a laser. On a laser absorbing layer, a bioink layer is coated. The laser is directed on the absorbing layer, which will store the energy creating a heated area that generates a hydrogel bubble that falls to the substrate by gravity. Conversely to the extrusion process, the inkjet, drop-on-demand, and laser-assisted printing are non-contact extrusion systems. This allows minimizing the potential cross-contamination between the extrusion nozzle and the substrate [14, 15], and this is important for future development of the technique for *in vitro* bioprinting applications.

Stereolithography

Directly translated from the 3D printing industry, SLA methods have been applied to 3D bioprinting. Using a laser that excites a photopolymer such as gelatin methacrylate, or alginate methacrylate, the hydrogel is crosslinked using non-cytotoxic radical activators. Using the methacrylate chemistry, iterations of this technique are pursued to apply DLP to 3D bioprinting. The DLP technique permits to gain speed and resolution to create complex shapes made of living materials [16, 17].

Electrohydrodynamic

The development of advanced electrospinning technologies, has made it possible to apply this method to bioinks. Although the methods use high voltage, because the distance between the nozzle and collector plate is low, the current can be kept minimal to have no impact on the cell viability. By changing the current, viscosity of

the bioink, and the distance between the nozzle and plate, the bioink can be deposited as a droplet or as a continuous filament. Bioink formulations have been proposed based on gelatin methacrylate and alginate. While this technique is quite promising in terms of resolution, it still suffers limitations on the height of the object and size of the printed living materials [18, 19].

Needle array

Spheroids dispersed in a hydrogel can be printed using extrusion-based techniques, but this requires the use of biomaterials [20]. Yet, we have seen that spheroids are particularly useful for biomaterials-free manufacturing approach as they can themselves generate the needed biomaterials to maintain their mechanical structure. The needle array manufacturing technique or Kazan permits a biomaterials-free 3D bioprinting approach. The spheroids are skewed on a needle array that is patterned in the desired shape. Layers upon layers, the spheroids are positioned on the needles. The spheroids are then incubated, and fused with each other. Once the tissue is matured, the needles are removed, and a final 3D shape is obtained. This method can be automated, and different shapes can be obtained by changing the array pattern. This approach enables to grow a tissue mass rapidly [21] (Figure 9.8).

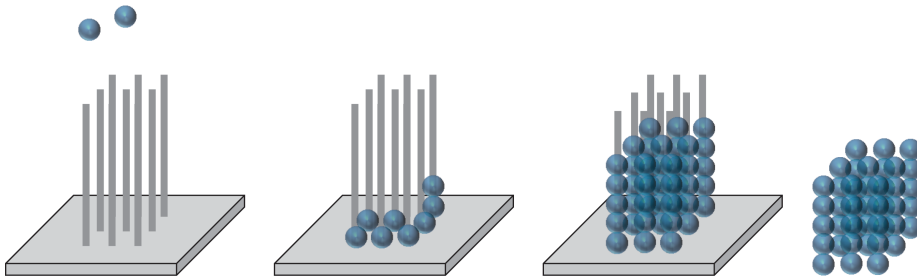


Figure 9.8: A needle array is used to organize spheroids into the desired shape. Then, the needles are removed and the spheroids fuse as the cells grow and generate an extracellular matrix.

Freeform reversible embedding of suspended hydrogels

The complexity of objects manufactured by extrusion 3D bioprinting is limited by gravity. If printed on a planar substrate, an overhang cannot be created without support to avoid the object to collapse on itself. To overcome this limitation, the freeform reversible embedding of suspended hydrogels (FRESH) technique permits to print directly into a hydrogel that behaves like a Bingham plastic, at low stress, it is a solid, and at high stress, it is a liquid. This means that when the extrusion nozzle moves within the FRESH bath, it is under stress and the hydrogel bath behaves like a liquid. Once the bioink is deposited, there is no longer stress applied to the hydrogel bath, so it acts like a solid and can support the bioink mass. Usually, the FRESH bath is made of gelatin.

So that once the bioink is deposited, the gelatin bath can be removed by heating the bath thus melting the gelatin. To obtain a Bingham plastic with gelatin, it must be processed into a particulate that forms an assembly of small gelatin beads. This method has been applied to different bioinks and is also used for silicon printing in a poly (acrylic acid) microparticulate bath. This approach can be used to scale up the bioprinting process as the impact of gravity is removed and thus the risk for the printed structure of collapsing [22].

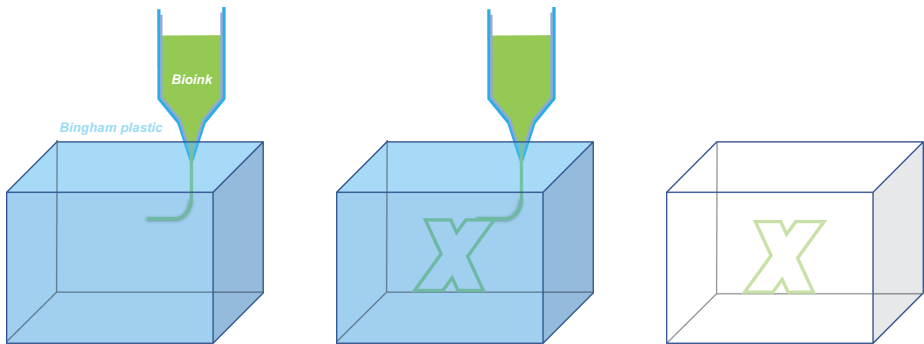


Figure 9.9: FRESH process: the hydrogel-based bioink is deposited in a support media (blue) that maintain the ink in place, after the printing, the media is removed.

Sacrificial hydrogel

Hollow geometries cannot be printed when using an extrusion bioprinting approach because adding material above a hole is not possible. To overcome this limitation, in FDM 3D printing support materials are added. Similarly, in extrusion 3D bioprinting, a sacrificial support hydrogel can be used. Channels can be manufactured using a bioprinter with two printer heads, one for the bioink, one for the sacrificial hydrogel. The sacrificial hydrogel is used to fill the channel. Then, the sacrificial hydrogel is removed, leaving a hollow tube. The typical sacrificial hydrogel used for this sort of bioprinting is Pluronic that has an LCST. So, the part can be printed at room temperature, and when incubated at 4 °C, the Pluronic becomes liquid and can be washed out of the channel. Once the hollow channel is obtained, it can be used to simulate blood vessels [23] (Figure 9.10).

9.5 *In vivo* 3D bioprinting

With the development of medical robots and the introduction of surgical robot arms that a surgeon (for now) controls, the potential of *in vivo* 3D bioprinting is slowly emerging. Surgical robotic arms are now proposing a solution for remote surgery and more precise key-hole procedure. This paves the way toward the deposition of materials with regenerative or reparative potential during the surgery. To achieve this, 3D bioprinters are now

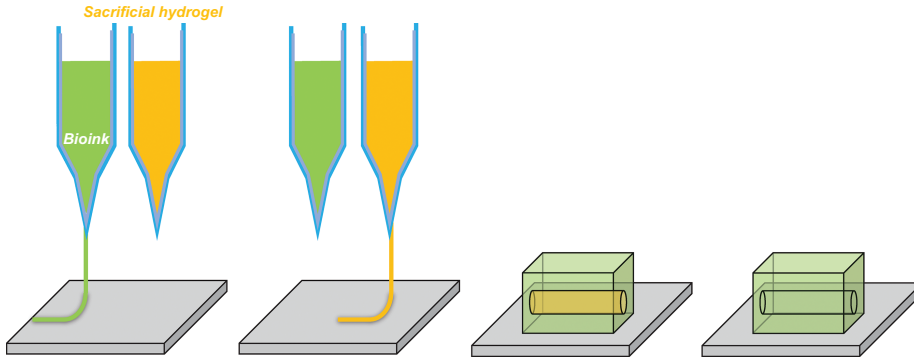


Figure 9.10: Sacrificial hydrogel (yellow) is printed alternatively with the bioink (green). Once the multimaterials object is printed, the sacrificial hydrogel is removed by activating an external stimulus, lowering the temperature below 4 °C for Pluronic.

transitioning from a 3-axis geometry to a 6-axis arm architecture (Figure 9.11). As a result, more freedom of movement permits access to difficult areas and perfectly follows anatomical features.

The current proposal for *in-vivo* bioprinting consist of a workflow that first incorporates a scanning step to acquire the geometry of the area to be treated and the size of the pathology. For instance, to treat a bone fracture, scanning of the bone and its fracture is first needed. Then, the robotic arm changes the tool and uses an extrusion nozzle to deposit a bioink onto the fracture. The bioink, once put in place, is expected

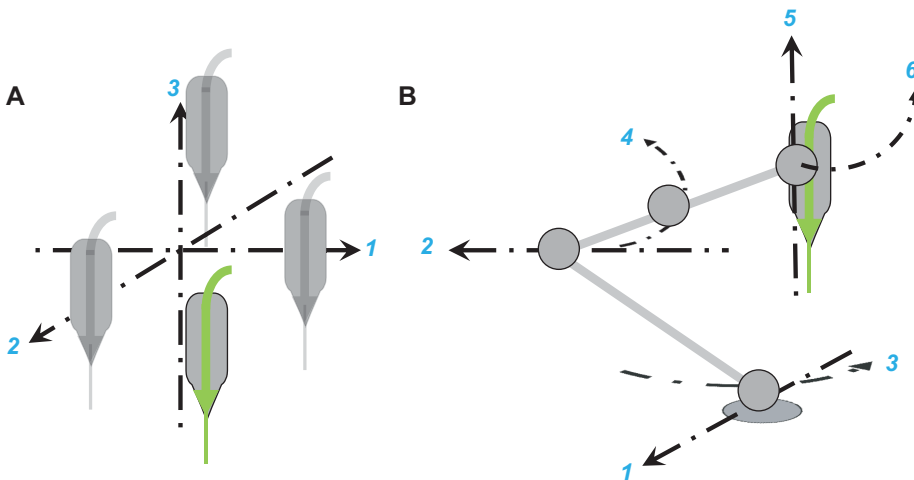


Figure 9.11: Comparison of the movement of freedom of (A) 3-axis printer with (B) 6-axis printer.

to have regenerative capabilities to improve the healing time and quality (Figure 9.12). This scenario is far from fiction, and technological proof-of-concept is being conducted. Beyond the engineering challenge, the outcome of such procedures is measured by the biological and medical benefits. To be adopted, automated surgical procedure must provide a cost, safety, and performance benefit for the patients [14, 24].

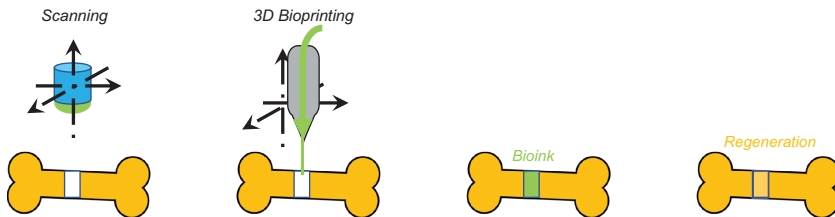


Figure 9.12: Proposed workflow for the introduction of bioprinting for medical procedures: first scanning of the pathology, deposition of a bioink, the bioink fits the pathology and provides a medical benefit such as better repair or regeneration of the tissue.

9.6 Pharmaceutical 3D printing

One of the applications for 3D printing and 3D bioprinting is the potential application for a patient-specific pill that could contain several active pharmaceutical ingredients (APIs). For a patient who must get several drugs per day, having a pill that contains different ingredients could be beneficial. When incorporating several drugs in one pill, not all the ingredients will be released at the same speed and amount. For instance, API positioned at the surface of the multi API pill will be released quicker than the one positioned inside of the pill. Here, the surface area and the material used to make the pill are critical to obtaining the required drug release profile. For instance, a high surface area means a quicker release, but biomaterials that quickly degrade in the stomach also give faster release. So, the geometry and materials need to be precisely adjusted for each formulation. Further application of this technology might be developed for drugs that are dosed differently for each patient. For instance, in pediatric, APIs doses are given as an amount per body mass. Because pediatric patients' mass greatly varies, their treatment is prepared by the in-house pharmacy in hospital settings. Using an AM approach has the potential to create an attractive shape with the required dose to facilitate the willingness of patients in taking the medicine while being versatile to be adapted for each dose [25, 26] (Figure 9.13).

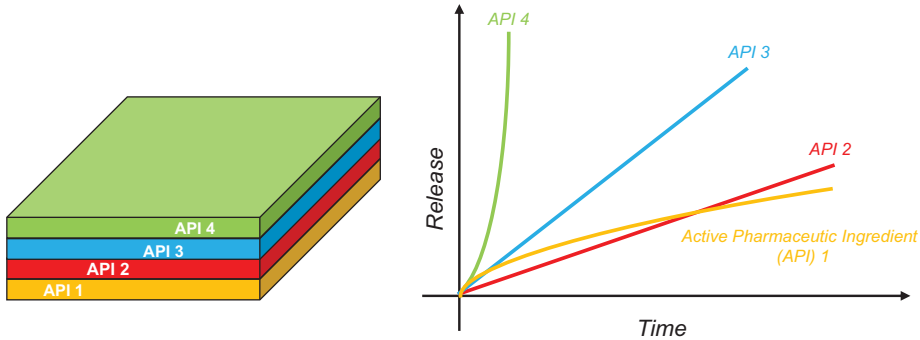


Figure 9.13: 3D printing of pharmaceutical pill containing multiple active pharmaceutical ingredients (API) to obtain multiple drug release profiles in one formulation.

9.7 4D Printing

Introducing the fourth dimension to the 3D printing process means that the part once printed will evolve with time upon exposure to an external stimulus. Using materials that are reactive to humidity, magnetic field, light, or pH, the shape of the printed object can evolve predictably. For instance, using hydrogel that responds to temperature objects that fold when exposed to a change of temperature can be designed. Such printed parts have the potential to be used for drug delivery when the drug carrier must change shape after implantation (Figure 9.14).

Because they are living materials, most of the 3D bioprinted objects are changing over time. Cells are replicating, secreting extracellular materials, or degrading the initial hydrogel matrices. Leveraging the predictable fate of cells, 4D printing concepts can be used to guide living cells into mature tissue replicate to stimulate

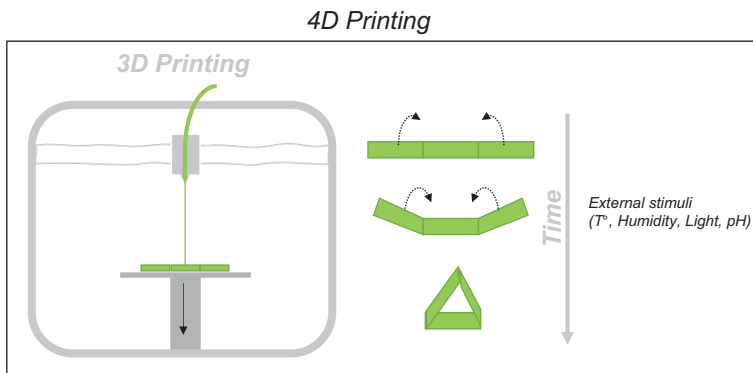


Figure 9.14: 4D printing concept. An object is 3D printed, and by getting exposed to external stimuli, the shape of the objects is changing.

their replication, differentiation, or matrices degradation capabilities. Yet, these concepts are in their infancy and more fundamental research needs to be carried out to transform these concepts into products [27, 28].

9.8 Imaging 3D object

Manufacturing 3D living objects by bioprinting requires the ability to observe the evolution of these living objects once printed. Yet, imaging tridimensional objects is challenging, but several techniques are now available (Figure 9.15).

9.8.1 Widefield microscopy

The widefield microscope uses a series of glass lenses to magnify the observed object. The light beam goes into a series of convex lenses that enlarge the image. Usual magnification ranges from 10x to 100x with a resolving power of about 200 nm. Unfortunately, this type of microscopy is not well suited for acquiring 3D images. So, 3D printed samples are usually sectioned into thin layers of materials deposited on a glass slide. If biological information needs to be obtained, then staining with antigen (immunohistology) or dye (histology) is needed. The usual dye that is used can target specifically the nuclei (Hoechst) or the actin skeleton (fluorescently - tagged phalloidin). While there is a constant improvement of fluorescent dye, most of them require crosslinking the proteins (fix) and permeabilizing the cell membrane to allow the dye to reach its target.

9.8.2 Laser microscopy

Using a laser instead of a light beam permits to selectively shine light on the sample and reduces the signals from unfocused parts. By using a point illumination method and a pinhole in front of the detector, out-of-focus signal can be reduced. It is then possible to gain a high resolution in the depth (height) of the sample so as to only acquire a signal from one focal plane. A 3D image of the sample can be gathered by scanning different focal planes. Here, only fluorescence information can be obtained and thus the sample needs to be stained with antigen (immunofluorescence) or fluorescently-tagged toxin such as phalloidin. While fluorescent dye that can be given to a living specimen without altering its physiology are available now, they are limited. If specific proteins are needed, the sample needs to be fixed and permeabilized to be marked with fluorescently-tagged antibodies. However, laser microscopy does not require sectioning of the specimen. Alternatively, genetically modified cells that expressed protein directly as a fluorescent protein like the green fluorescent protein can be used to follow the cell fate and function. Improvement of the laser microscopy

technique has been made with a spinning disk that allows fastening the technique by using a rotating disk punched with holes that let the light go through at regular intervals.

9.8.3 Light-sheet microscopy

The light-sheet microscope uses a laser beam that is focused in one direction. Doing so illuminates only the observed section. Like the confocal, it reduces the photobleaching that can occur by the over exposition of the sample like in the widefield microscope. This is particularly appreciated when imaging living animals as it reduces the acquisition time and thus the stress on the animal. Instead of using points like in the confocal but a light sheet, the image acquisition is faster than the confocal while giving a good contrast. In biology, this technique is used with genetically modified animals that produces the protein of interest with an attached fluorescent protein.

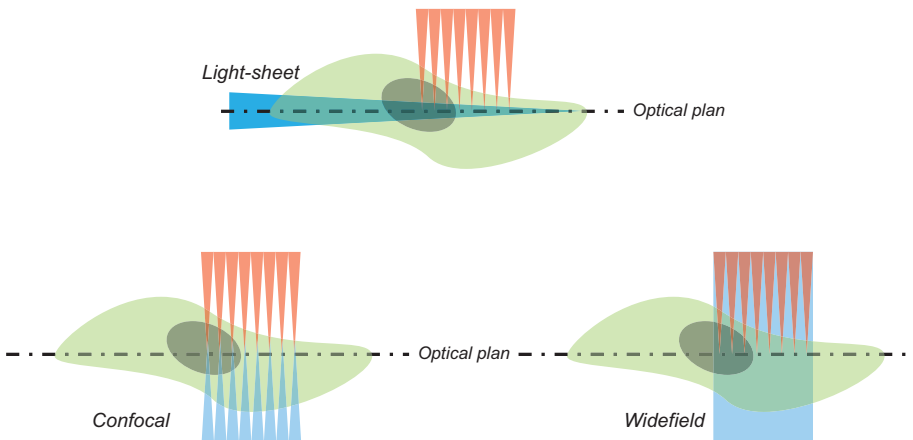


Figure 9.15: The three principle microscopic techniques used in biofabrication. The **light-sheet** gives a rapid sampling time, the **confocal** gives high resolution but can take long, and the **widefield** microscopy has a contrast limitation as all the sample is illuminated at once.

9.8.4 Tomography

In light microscopy, to obtain a 3D image of an object, it is needed to physically section the object into thin slices. The slices are mounted on a glass slide, and images of the slices of the objects are acquired. The object can then be reconstructed into a digital 3D image by combining all the individual images together. With a technique that permits to go through the matter such as x-ray, MRI, and fluorescence, images of the

inside of the object can be obtained. Using a tomography approach, imaging an object by section enables the reconstruction of a 3D digital image of the sampled object.

X-ray

X-rays are electromagnetic penetrating radiation with a wavelength between 10 pm and 10 nm. These radiations can go through many materials and are well suited to obtain anatomical information of living animals. For 3D printing and bioprinting, they permit to get precise 3D reconstruction of the object and obtain a 3D digital image that can be compared to the intended design. Initially restrained to imaging anatomy, the development of an instrument combining X-ray spectroscopy (to get chemical information) with imaging offers a promising potential to use X-ray for both anatomical and physiological characterization of the tested specimens. For example, in some medical applications of X-ray, contrast agents such as barium sulfate are ingested by the patient to increase the contrast of the digestive tract. Barium sulfates concentrate in the digestive tract, thus increasing the density of the tissue and permitting to obtain a better image.

Magnetic resonance imaging

Using magnetic field gradients and radio wave, images of living specimens anatomical and physiological process can be obtained. In comparison to X-ray, which is best for hard tissue, MRI is better suited for soft tissue imaging. Based on the magnetic resonance of hydrogen nuclei, a radio frequency pulse is used to excite the electrons of an atom, and the resulting signal of this excitation is collected with magnetic receiving coils. For some applications, contrast agents are required to improve the image. This consists of atoms that alter the electromagnetism of the surrounding water and thus obtain a better contrast of the observed tissue.

9.8.5 Scanning electron microscopy

Obtaining high-resolution imaging of an object often requires using electron microscopy. Instead of using photons, electrons are shot onto the specimen, and the scattered electrons are collected, giving an image of the sample. Additionally, if the instrument is equipped with an x-ray detector, information on the atomic composition of the sample can be obtained. In the scanning electron microscopy process, non-conductive samples must be coated with a thin conductive metal layer such as gold. The electron beam scans the surface of the sample, and a full image of the surface is obtained with depth contrast. The stage can be tilted, but the result is a planar 2D picture. Because the instrument operates in a high vacuum (to get the electron beam), the samples need to be crosslinked and dehydrated to avoid shrinking and change of dimension under vacuum.

Table 9.2: Commonly used imaging techniques for 3D samples.

Technique		Sample preparation	Resolution	Damage	Cost		Image
					Sample	Machine	
<u>Microscopy</u>	Light	Sectioning	μm	Yes	\$	\$	2D
		GMO/live staining		No			
	Confocal	Immunostaining	μm	Yes	\$\$	\$\$	3D
		GMO/live staining		No			
	Light-sheet	GMO	μm	No	\$\$	\$\$	3D
<u>Electronic</u>		Fixed/coated	nm	Yes	\$	\$\$\$	2D
<u>Tomography</u>	X-ray	Contrast agent	μm	No	\$	\$\$\$	3D
	MRI	Contrast agent	μm	No	\$	\$\$\$	3D

9.9 Quiz

1. What was the first 3D printing technique developed, and when?
2. What was the first application of 3D printing in medicine?
3. Which 3D printing technique gives thermoset-made materials, which one a thermoplastic?
4. What polymeric materials are used for the 3D printing of implants?
5. What materials are used for bioprinting?
6. What is a bioink?
7. What are the main bioprinting technologies?
8. What are the main 3D printing technologies?
9. What is 4D printing?
10. What are the two main advantages that 3D printing of pharmaceutical pills could bring?



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10 Applications of biofabrication

10.1 The start of an industry

With the growing range and complexity of available biofabrication techniques, we are getting closer to manufacturing synthetic organs. The initial aim of biofabrication was to address the transplantation organ shortage. But to achieve this, we need to be able to manufacture fully functional organs constituted of several tissues, which is a highly complex task. While the current advances in the field have permitted a significant breakthrough, new opportunities have emerged in unexpected areas. Now biofabrication methods are applied to generate tissue for drug and cosmetic testing, meat production, and living materials for architecture and design.

10.1.1 Tissue model

Three main factors are putting pressure on pharmaceutical drug development. The first one is an aging society. The United Nations predicts that in 2030 over 10% of the world-wide population will be over 65 years old. An aging population means more severe pathologies will occur and an increasing need for new medicines to sustain a comfortable life. In addition, there is legal pressure that will impact how pharmaceutical research and development is carried out. In 2009, the European Union banned animal testing for cosmetical research and development. In 2010, a directive followed up to draw a roadmap to replace laboratory animals with alternative methods, including engineered tissue. Finally, economic pressure is pushing to change how pharmaceuticals are developed. Indeed, drugs are not discovered by serendipity anymore, and the cost of the industrial research of pharmaceutical molecules has nearly doubled in the last decade. So, methods to reduce the cost of development are needed, and biofabricated tissues are one promising technology. Biofabricated tissue is expected to play a critical role in drug development and replace animal testing. Tissue models engineered with human physiology are expected to provide a closer model to human biology than animal models, thus providing an alternative to animal testing.

10.1.2 Meat production

With the exponential increase of the world population and the need to access cheap protein sources, animal farming has become industrialized, sometimes unethical, and a significant producer of greenhouse gases. One technological solution to the protein food supply currently in development is to grow meat in laboratories, the so-called cultured meat. Using current biofabrication techniques, cost reduction

in cultured meat production is in the making. But only growing the cells is not enough. To reproduce the texture and flavors of the natural meat, tissue organization must be mimicked. Indeed, natural muscle for meat consumption is marbled by fat tissue, which creates a particular taste upon cooking. Here biofabrication techniques are needed to organize the myocyte and adipocyte cells into the meat [1].

10.1.3 Living materials

To reduce animal farming, endeavors to create laboratory leather are explored. Two approaches are investigated: one to use animal cells to produce collagen and form animal-based leather [2], the second approach uses fungi and biomaterials to reproduce the materials properties of natural leather [3]. Using a combination of biomaterials with fungi or bacteria to engineer living materials [4] have been used to make packaging materials. The fungi are grown on a support. As the fungi develop, it creates a mycelium that forms strong materials [5]. A similar approach can be used to make cellulose using bacteria that naturally produce the polysaccharide. Upon inactivation of the bacteria, a strong and stable material is obtained. By shaping the growing support of the bacteria, complex shape and design of the bacterial cellulose can be achieved [6].

10.2 Industrial processes

For biofabrication to be utilized to manufacture products, it has to follow the regulation and guidelines to ensure the safety of biomedical materials. For the north-American market, medical devices and pharmaceuticals need to be produced following good manufacturing practices (GMP), which are a set of guidelines that provide for systems that assure proper design, monitoring, and control of manufacturing processes and facilities. It encounters the quality management system, personal training, facilities management, and product control. Next, automated systems utilized for manufacturing or quality control need to have an electronic signature that ensures that the electronic records are considered trustworthy and reliable as much as paper records. This limits the risk of falsification of electronic records. These two standards are brought to use to ensure the manufacturing process's safety and traceability. For medical devices (that could be biofabricated) a system to monitor the quality must be brought in place. These requirements are set into the ISO 13,485 norm. This norm is necessary for the obtention of approval for the commercialization of medical devices in the European Union and associated countries. Because there are rules and regulation that needs to be followed to ensure the safety and quality of medical devices, if biofabrication methods are required to manufacture such devices, these methods must follow the specific guidelines. Meaning that the computer and instruments utilized in the biofabrication process (electrospinning, 3D bioprinter as an example) must complain to the electronic

documentation regulations, and the manufacturing process must follow a quality management system [7].

10.3 Sterilization

Finally, biofabricated medical devices must be produced without pathogens and microorganisms. Two manufacturing approaches can achieve this: manufacture in non-sterile conditions and then sterilize the device at the end of the process, or manufacture under aseptic conditions. Meaning the whole manufacturing process is done under sterile conditions. Usually, the first strategy is preferable for cost-associated reasons, but it is not possible to have final sterilization in some cases. For example, if living cells are incorporated into the medical device, no sterilization methods can be used. Three main techniques are available to sterilize medical devices: heat sterilization, chemical sterilization, and radiation sterilization (Figure 10.1).

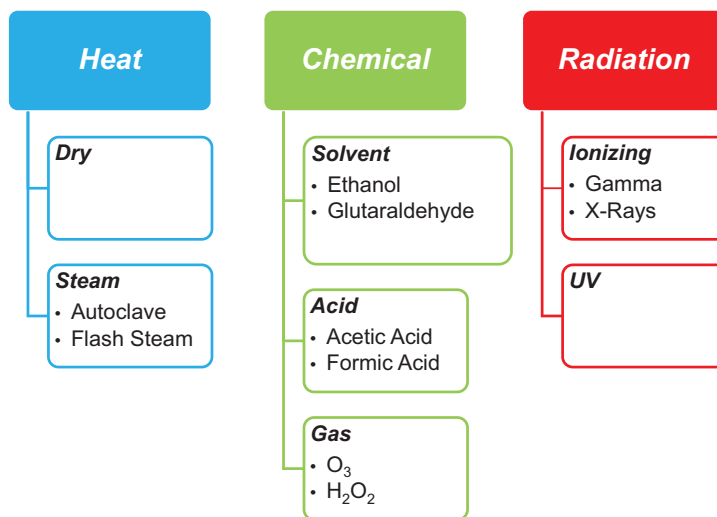


Figure 10.1: Sterilization methods commonly used in biofabrication.

In contrast, heat and radiation are preferred because they can be easily scaled up and do not use toxic chemicals that must be removed. Heat sterilization usually consists of steam sterilization (autoclave) that heat the samples above 120 °C for several minutes. This technique might not be compatible with all polymers, especially those with melting temperature below the steam temperature. Radiation sterilization is the preferred method for poly(styrene) sterilization. The radiation does not induce heat and can go through packaging. Therefore, it allows for the sterilization of many manufactured and packaged products, especially plastic ware used for the *in vitro* cell culture. For some

polymer systems, the radiation generated during the process can induce unwanted crosslinking of the polymers and modify the product's properties. In another case, the radiation can induce crosslinking of the polymer and further improve the mechanical properties. Finally, the efficiency of the sterilization process must be assessed and proven to ensure the safety and quality of the produced device.

10.4 Quiz



1. Why could tissue models reduce the use of animal models in pharmaceutical development?
2. What are the three main sterilization methods?
3. What are the three factors pushing toward a change in pharmaceutical development?
4. What is aseptically manufacturing?
5. What is the significant risk of radiation sterilization?
6. What is the major risk of heat sterilization?
7. What are the factors pushing toward laboratory meat production?
8. What are the three main regulatory frameworks for medical device manufacturing?
9. Give an example of an application for living materials.
10. When was banned animal testing of cosmetics in Europe?

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Index

- 3D bioprinting 153
- 3D cell cultures 153
- 3D printing 148
- 4D printing 161

- acoustic wave 15
- acoustic waves 23
- active pharmaceutical ingredients 106
- active pharmaceutical ingredients (APIs) 160
- additive manufacturing (AM) 147
- agarose 19, 68, 154
- alginate 67, 124, 154
- and rotary cell culture system (RCCS) 20
- animal testing 106
- atactic 30
- autoclave 171

- beads 21
- bioactive glass 46
- biofabrication 8
- biomacromolecule 80
- biomacromolecules 85
- biomaterial 27
- biomimicry 137
- bioprinter 135
- bioreactors 12
- block copolymer 121
- blood vessel 138

- calcium phosphate 45
- cancer 24
- Capillary molding 102
- cell adhesion 12, 87, 91
- cell adhesion peptides (CAPs) 87
- cell infiltration 129
- cell sheet 16
- cell sorting devices 105
- cellulose 67
- chemotaxis 144
- chitosan 71
- click chemistry 63
- CO₂ 120
- coaxial extrusion 124
- cobalt–chromium 48
- collagen 81, 154

- computer numerical control (CNC) 147
- computer-assisted software (CAD) 141
- contact angle 92
- convection effect 117
- critical translational speed (CTS). 128
- crosslinking 55
- crystal 116

- decellularization 85
- design 135
- dextran 69
- Diels–Alder 63
- digital light processing (DLP) 150
- droplet 119
- drop-on-demand 155
- drug release* 35
- drugs discovered 105
- dry spinning 122
- durotaxis* 144

- electrohydrodynamic (EHD) 156
- electrospinning 125
- emulsions 23
- extracellular matrix 11, 79
- extracellular matrix (ECM) 91
- extrusion 156

- fiber bonding 123
- fibroin, Silk 72
- fibronectin 81
- Flory–Huggins 121
- foam 115
- Fourier-transformed infrared spectroscopy 97
- freeform reversible embedding of suspended hydrogels (FRESH) 157
- freeze-drying 119
- functionalization (PDMS) 51
- fuse deposition modeling (FDM) 149

- gelatin 72, 154
- gelatin methacrylate 154
- gelatine methacrylate (GelMA) 62
- Gellan gum 70
- genipin 61
- glass transition* (T_g) 28

- glutaraldehyde 60
- glycosaminoglycans, GAGs 80
- good manufacturing practices (GMP) 170
- hanging drop 18
- haptotaxis 145
- hollow 124
- hydrogel 55, 86, 102
- immune response 42, 129
- in vivo 3D bioprinting 158
- inkjet 155
- inorganic biomaterials 45
- in silico* design 143
- integrin 81
- Irgacure 2959 62
- ISO 13485 170
- isotactic 30
- Kazan 157
- Kenzan 25
- laminar flow 104
- laser-assisted 156
- leather 170
- Living materials 170
- lower critical solution temperature (LCST) 16
- M/G ratio 67
- macrophage 129
- magnetic 22
- magnetic resonance imaging (MRI) 164
- matrix metalloproteinases 81
- Meat production 169
- mechanical heart 3
- medical resonance imaging (MRI) 151
- melt electrowetting 128
- melt spinning 122
- melting temperature* (T_m): 28
- metal alloys 48
- metal printing 152
- metal-assisted chemical etching (MACE) 99
- Michael addition 63
- microbeads 105
- microcontact printing 103
- microfiber 121
- microgravity 20
- microscope 162
- microtransfer molding 103
- molds 15
- morphogenic 16, 140
- multijet fusion (MJF) 150
- nanocellulose 154
- nanoneedles array 101
- nanoparticles 22
- nanopillars 100
- nanowires 100
- needle array 157
- network 55
- nitinol 48
- non-adhesive 15, 19
- organ regeneration 4
- organ systems 7
- organoids 24
- organ-on-a chip (OOC) 105
- organs transplantation 5
- organs-on-a-chip 108
- parametric equation 143
- PCL 149
- peacemaker 3
- pediatric 160
- PEEK 149
- pharmaceutical 3D printing 160
- pharmaceutical drug development 169
- pharmacodynamic 107
- pharmacokinetic 107
- phase diagram 119
- phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) 62
- photocross-linking 61, 63
- photoinitiator 61
- photolithography 98
- physical hydrogels 65
- pill 160
- pixel 142
- PLA 149
- plasma 92
- plasma polymerization coating 96
- plasma polymerize 94
- plastic printing 152
- Pluronic 154, 158
- poloxamer 65
- Poly (ethylene glycol) 58
- poly(2-hydroxyethyl methacrylate) 19

- poly(ethyl vinyl acetate) 34
- poly(lactic-co-glycolic acid) 38
- poly(NIPAAm) 16
- poly(*N*-isopropyl acrylamide) 16, 19
- poly(styrene) (PS) 92
- poly(propylene fumarate) 41
- polyacryl amide 57
- polyacrylic acid 60, 158
- polyamide 33, 149
- polyanhydride 40
- polycaprolactone 38
- polycarbonate 33
- polydimethylsiloxane 49
- polyelectrolyte 60
- Polyether ether ketone 36
- poly(ether sulfone) 44
- polyethylene glycol 19
- polyglycolic acid 36
- polyhydroxy alkanoates 43
- polyjet (PJ) 150
- polylactic acid 37
- polymer melt 118
- polyphosphazenes 51
- polypropylene 30
- poly(propylene fumarate) 41
- polysaccharide 67
- polystyrene 12
- polytetrafluoroethylene 32
- polyurethane 42, 115
- polyvinyl alcohol 40
- polyvinyl chloride 33
- polyvinyl-pyrrolidone 59
- pore 113
- pore size 129
- porogen 115
- porosity 113
- Prometheus 4
- prosthetics 2
- protein cross-linking 60
- proteoglycans 81
- Radiation 171
- replica molding 101
- resolution 142
- Reynolds number 104
- ring-opening polymerization 39, 50
- sacrificial hydrogel 158
- salamander 1
- salt 116
- scanning electron microscopy (SEM) 164
- selective laser sintering (SLS) 149
- soft lithography 101
- solvent 118
- spheroids 18, 157
- stem cells 22
- stereolithography 156
- stereolithography (SLA) 148
- Sterilization 171
- strand promoted 65
- supercritical fluids 120
- surface chemistry 95
- syndiotactic 30
- Tagliacozzi 4
- temperature-responsive 19
- tensiotaxis* 144
- T_g 120
- thermoplastic* 28
- thermoreversible 68, 70
- thermoset* 28
- tissue engineering 6
- titanium 49
- Tityus 4
- tomography 163
- transplantation 7
- trypsin 18
- turbulent flow 104
- two-photon polymerization 63
- user-centered 138
- vectors 142
- wet spinning 122
- wettability 92, 96
- Xanthan gum 70
- X-ray 151, 164
- X-ray photoelectron spectroscopy (XPS) 96
- Ziegler and Natta 29
- Ziegler–Natta 31

